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# FINAL REPORT 2024

**SA WINE number : SAGWRI BD 20-01**

## 1. PROGRAMME & PROJECT LEADER INFORMATION

| | Research Organisation leader | Project leader |
|---|---|---|
| **Title, initials, surname** | Prof MA Vivier | Prof Benoit Divol / Dr Evodia Setati |
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## 2. PROJECT INFORMATION

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|---|---|
| **Project title** | Does intracellular coenzyme availability limit yeast impact on wine flavours? |
| **Short title** | Yeast redox status and metabolic fluxes |

| | | | |
|---|---|---|---|
| **Fruit kind(s)** | Grapes | | |
| **Start date** (mm/yyyy) | 2021-01 | **End date** (mm/yyyy) | 2023-12 |

| | |
|---|---|
| **Key words** | Yeast, redox status, flavour compounds, wine |

| | CFPA | RAISIN SA | HORTGRO | SATI | SA WINE | ARC | OTHER |
|---|---|---|---|---|---|---|---|
| **TOTALS All years** | R 0 | R 0 | R 0 | R 0 | R 1084325 | R 0 | R 999999 |

| | |
|---|---|
| **Total cost of entire project** | R 2084324 |

## 3. EXECUTIVE SUMMARY


**Objectives and Rationale**

This project investigated how different wine yeast species and strains manage redox cofactors during alcoholic fermentation and the influence thereof on flavour compound production. By monitoring redox dynamics and assessing environmental factors, it identifies conditions winemakers may manipulate to potentially enhance yeast persistence and impact on wine characteristics.

**Methods**

Fermentations were conducted using synthetic grape juice to monitor redox status and metabolite production in various yeast species and strains. A multifactorial experiment assessed the impact of environmental factors, and sensory evaluations were performed on real grape juice fermentations. Next-generation sequencing compared gene expression under oxygenated and unoxygenated conditions.

**Key Results**

A lack of $NAD^+$ regeneration leads to yeast's premature decline.

Mechanisms to regenerate $NAD^+$ differ between yeasts resulting in unique metabolic footprints.

Nicotinic acid deficiency impacts redox balance and fermentation performance.

Thiamine deficiency impacts fermentation rate.

Oxygen could be a lever to alter metabolic footprint and sensory attributes in wine.

**Key Conclusion of Discussion**

Unlike *S. cerevisiae*, certain non-*Saccharomyces* species struggle to ferment due to insufficient NAD+ regeneration, leading to premature decline. Each yeast's distinct redox metabolism results in different metabolite production and fermentation performance. Nicotinic acid deficiency results in poor fermentative efficiency and altered metabolic footprint. Oxygenation could be used to impact fermentation kinetics and wine properties, demonstrating its potential as a lever to modulate yeast performance and wine characteristics. Thiamine and vitamin B3 supplementation had minor impacts on wine composition and sensory profiles in real grape juice, likely due to sufficient initial vitamin levels.

**Take Home message for Industry**

Yeast strain selection and ensuring adequate nicotinic acid levels are crucial for optimal fermentation and flavour production. Controlled oxygen exposure can alter wine properties and sensory attributes without causing oxidation or sensory defects, providing a valuable tool for winemakers to influence wine style. This research provides insights for optimizing yeast nutrient formulations and fermentation conditions to enhance wine quality and consistency, particularly for non-*Saccharomyces* yeasts.

## 4. PROBLEM IDENTIFICATION AND MOTIVATION

**Problem Identification**

Yeast species, and even different strains within the same species, have unique ways of responding to environmental conditions during the fermentation of grape juice into wine. Factors including nutrient availability (i.e. sugars, nitrogen, and vitamins) and oxygen levels, play a role in shaping these responses. Alongside genetic factors, how yeasts react to these environmental factors affects the fermentation process and the overall quality of the wine. These responses lead to the production of various metabolites of interest which are tightly regulated by the balance of redox equivalents during fermentation. Yeasts may have different ways of managing their redox balance, which influences their behaviour in different environments. For example, some species are known for producing polyols like arabitol and sorbitol, along with aroma compounds while keeping acetic acid levels low. This could mean they struggle with maintaining their internal redox balance, but winemakers can use these unique metabolic traits to their advantage. This project aimed to monitor the redox status of various yeast species and explore which environmental conditions can be manipulated to boost the effectiveness and contribution of the yeast during winemaking.

**Motivation**

This project aimed to enhance our understanding of how metabolic fluxes in different yeast species affect the production of key oenological compounds, such as $C_5$–$C_6$ polyols, acetic acid, fatty acids, higher alcohols, and esters. It also explored how these fluxes influence yeast viability of non-*Saccharomyces* yeasts during fermentation. Investigating the impact of environmental conditions on these metabolic pathways will help provide winemakers with guidelines for optimising yeast exploitation. Recent studies from our group have shown that $C_5$–$C_6$ polyols produced by *Torulaspora delbrueckii* enhance mouthfeel, yet the mechanisms driving their production remain unclear. Some non-*Saccharomyces* yeasts are known for low acetic acid and high ester levels, while certain commercial *S. cerevisiae* strains produce excessive acetic acid despite their desirable aroma profiles. The underlying determinants of these metabolic differences are not well understood, but they are closely linked to intracellular redox status. This project will confirm this connection and offer strategies to improve the performance and persistence of various yeast species.

## 5. ACCUMULATED PROGRESS TABLE

| Objectives | Milestones (Significant event or stage in a project) | Date Achieved |
|---|---|---|
| Monitor redox status dynamics in selected yeast species throughout alcoholic fermentation and establish a link with a large variety of | Milestone 1: Monitor the intracellular redox balance of the above-mentioned yeasts throughout alcoholic fermentation in a synthetic grape juice medium.<br>Milestone 2: Attempt to model the production of categories of metabolite as a response to redox balance | 2022-06-30 |

| | | |
|---|---|---|
| metabolic sinks. | | |
| Evaluate the impact of various environmental factors on the yeast intracellular redox status and its dynamics throughout fermentation. | Milestone 1: A multifactorial design will be used to investigate all factors together and the statistical analysis will reveal which factors are the most impactful individually and/or collectively.<br>Milestone 2: The most impactful factors will be tested in upscaled fermentations to confirm the results obtained in Milestone 1.<br>Milestone 3: Milestone 2 will then be repeated in sequential inoculation (S. cerevisiae will be inoculated sequentially to non-Saccharomyces fermentations).<br>Milestone 4: Milestone 3 will be repeated in real grape juice. | 2024-06-30 |

## 6. WORKPLAN (MATERIALS AND METHODS)

Yeast strains used in this study:

*Saccharomyces cerevisiae* EC1118 (also referred to as Sc1 in some figures) (this strain was previously used in several projects so it was kept as a control).

*Saccharomyces cerevisiae* LMD14 (this strain was generated from EC1118 using directed evolution and produces more organic acids and glycerol and less ethanol and acetic acid).

*Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* LMD81 (high production of thiols, high production of volatile acidity, high nitrogen requirement).

*Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* LMD82 (this strain was generated from LMD81 using direct evolution and produces less acetic acid).

*Lachancea thermotolerans* Lt1 and IWBT Y1240 (high and low producers of lactic acid, respectively. Both produce low concentrations of acetic acid and high production of certain higher alcohols such as propanol and butanol.

*Torulaspora delbrueckii* Td1 (low production of acetic acid in high sugar musts and production of $C_5$ -$C_6$ polyols).

*Metschnikowia pulcherrima* Mp1 (production of a variety of fermentative aroma compounds)

*Kluyveromyces marxianus* IWBT Y885 (consumption of malic acid, high production of specific esters such as phenylethyl acetate).

*Starmerella bacillaris* MTF3768 and MTF3800 (high production of glycerol and fructophilic character).

### Milestone 1: Monitoring redox status and production of metabolite sinks in selected wine yeast species throughout alcoholic fermentation

Task 1: The different yeast species and strains mentioned above were inoculated in a synthetic grape juice-like medium and fermentations will be conducted in Erlenmeyer flasks under full anaerobic (for *S. cerevisiae* strains) or self-generated anaerobiosis (for non-S*accharomyces* yeasts). In a preliminary experiment, the fermentation kinetics and yeast biomass production were monitored. The goal was to determine at which time

points each strain reached the following physiological stages for sampling: 25%, 50% and 100% maximum yeast biomass, 30% 60% and 100% fermentation completion, where applicable. The 60% fermentation time was never reached in *M. pulcherrima* and only *S. cerevisiae* fermentations reached 100% fermentation completion (i.e. all sugars depleted).

At the set time points mentioned above, samples of culture were taken, and the yeast pellets were separated from the supernatants and quenched at -80°C in order to freeze the yeast metabolism at that point. Both quenched cell pellets and supernatants were frozen separately for further analyses (see tasks 2 and 3 below).

Task 2: From the yeast pellets, the intracellular content of $NAD^+$/NADH and $NADP^+$/NADPH were determined using enzymatic kits, namely MAK037 and MAK312 quantification, respectively (Sigma-Aldrich).

Task 3: In parallel to task 2, carbon sinks from the central carbon metabolism were determined using HPLC (Eyéghé-Bickong et al., 2012), and major higher alcohols, fatty acids and esters using GC-FID (Louw et al., 2009).

In order to further investigate the particular role played by lactic acid production on redox balance, *L. thermotolerans* strains were screened and characterised according to their fermentation dynamics and primary metabolite production, with particular focus on their ability to produce lactic acid. Additionally, the timing of lactic acid production and the impact of selected environmental conditions on the production of lactic acid were determined.

**Milestone 2**: **Evaluating the impact of environmental factors on the yeast intracellular redox status and its dynamics throughout fermentation.**

Task 1: A multifactorial design (Box-Behnken) was implemented to simultaneously test three factors and three different concentration levels (high, medium, low – mostly based on those typically occurring during grape juice fermentation). The three factors tested were tryptophan, thiamine and nicotinic acid. Each of these has been shown directly, or indirectly, play a role in the production of NAD and NADP. Fermentation dynamics were monitored, and the primary metabolites listed in milestone 1, task 3 were quantified at the end of fermentation (i.e. whenever $CO_2$ release ceases).

For *S. cerevisiae* strains only:

Task 2: The uptake of nicotinic acid, the intracellular concentration of nicotinic acid and intracellular NAD(H) were monitored over time in order to better understand the intracellular management of NAD(H) by the different yeast strains during fermentation.

Task 3: A transcriptomic analysis was performed using RNAseq to evaluate the transcription level of genes involved in NAD uptake and metabolism over time and under anaerobiosis/aerobiosis in the different yeast strains. The expression level of other genes was also assessed.

Task 4: The factors inducing the most extreme behaviours were applied to a new set of fermentations in real grape juice. This was tested in two strains namely EC1118 and LMD14. Fermentation kinetics, primary metabolites and the production of volatile aroma compounds were quantified to validate the results obtained in previous experiments in synthetic grape juice. Nicotinic acid was also added at the time when the strains reached their maximum

population to assess whether this had an impact on NAD management. Additionally, sensory

evaluation (using the Flash profile method, Valentin et al., 2012) was performed at the end of the fermentation to determine the drivers of the sensory differences and the consumers'

preferences. This milestone will be used to confirm the validity of the results obtained in

previous tasks/milestones in real grape juice and evaluate the potential application of the results in the wine industry.

## 7. RESULTS AND DISCUSSIONS

### Milestone 1:

Task 1: Preliminary experiments were conducted to determine the key time points to be considered. Figure 1 shows the time points considered against the backdrop of fermentation kinetics (shown as cumulative weight loss) and the population dynamics (shown as absorbance reading) in synthetic grape juice fermentation at 25°C. The time points selected cover important milestones in yeast physiology as well as the entire fermentation. For non-*Saccharomyces* yeasts, similar time points were considered (see below).

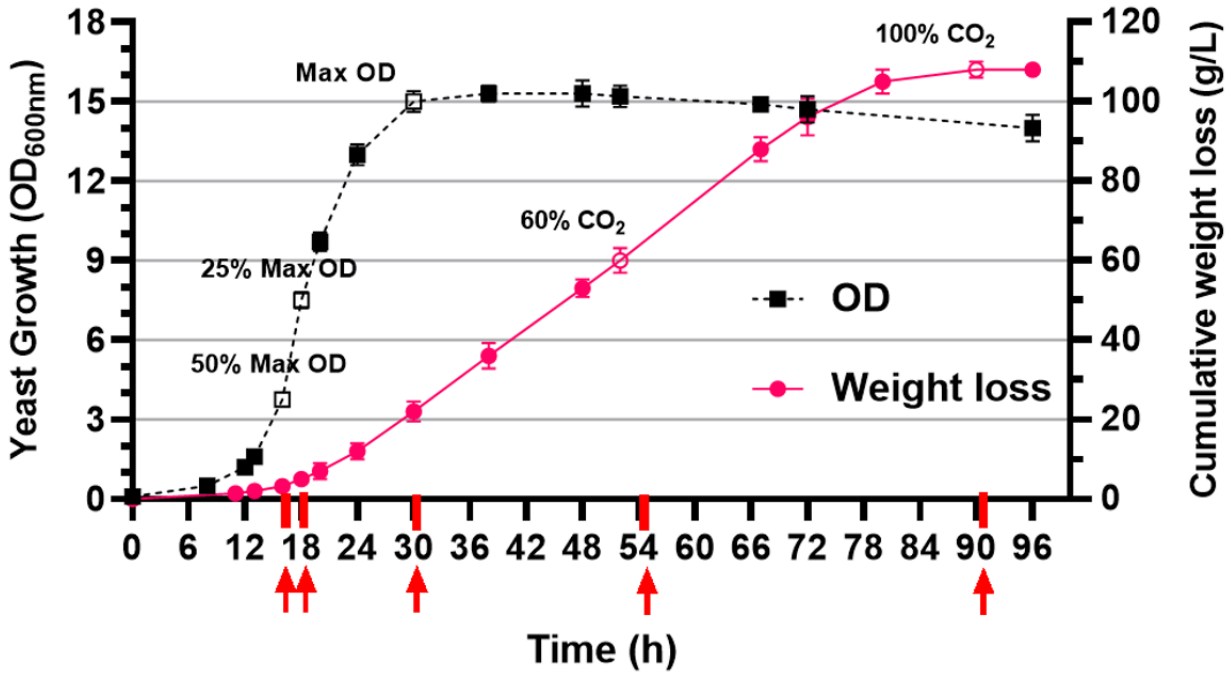


Figure 1: The typical fermentation kinetics (in accumulated weight loss) and the yeast biomass production (in absorbance) of a *Saccharomyces cerevisiae* strain. The sampling points chosen are indicated on the graph.

NB: In the experiments carried out on *S. cerevisiae* strains, all synthetic grape juice fermentations were conducted under strict anaerobic conditions to avoid oxygen interference with the yeast metabolism. Real grape-juice fermentations were conducted under normal cellar practices for white grape juice.
This experiment was repeated to determine the fermentation kinetics and population dynamics of all strains. The results for the *S. cerevisiae* strains are shown in Figure 2. Clear differences are observed between the four strains investigated. The related strains, LMD81 and LMD82 ferment the fastest, followed by EC1118 and LMD14. Despite the observed differences in absorbance readings, no significant differences were recorded for the actual yeast dry biomass at the end of fermentation. This was attributed to the differences in dry weights of the strains, as previously discovered for EC1118 and LMD14 (Lallemand, personal communication).

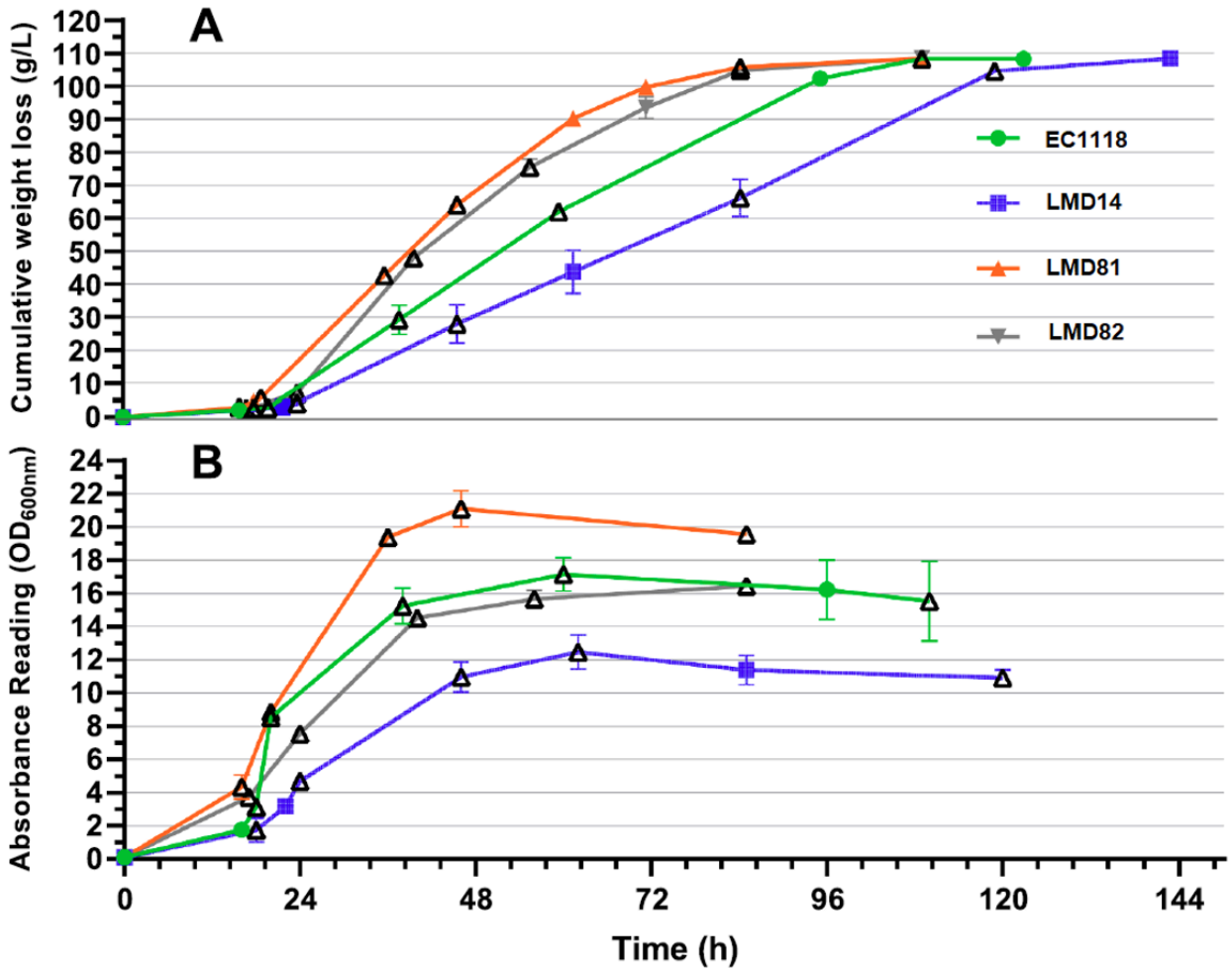


Figure 2: The fermentation kinetics and yeast growth for the four *S. cerevisiae* strains investigated. Sampling points are denoted by black triangles

NB: In the absence of oxygen, non-*Saccharomyces* yeasts do not grow. It was therefore not possible to carry out the fermentation under strict anaerobic conditions as for *S. cerevisiae*. For the non-*Saccharomyces* yeasts, the headspace was saturated with air and the conditions became progressively anaerobic as the fermentations proceeded. Since the non-*Saccharomyces* yeasts ferment at different rates and to different extents, it was decided to focus on the following sampling points: 50% growth, 30% and 60% fermentation completion, except for *S. bacillaris* and *M. pulcherrima* that do not reach the latter time point. The fermentation kinetics are shown in Figure 3. As expected, they differed widely between strains in terms of fermentation rate and extent of fermentation. All yeast strains displayed different patterns of fermentation kinetics that were similar to previous reports in literature. Except for *L. thermotolerans* and *T. delbrueckii*, the 50% growth did not align with the 30% fermentation completion time points in the non-*Saccharomyces* yeasts (data not shown).

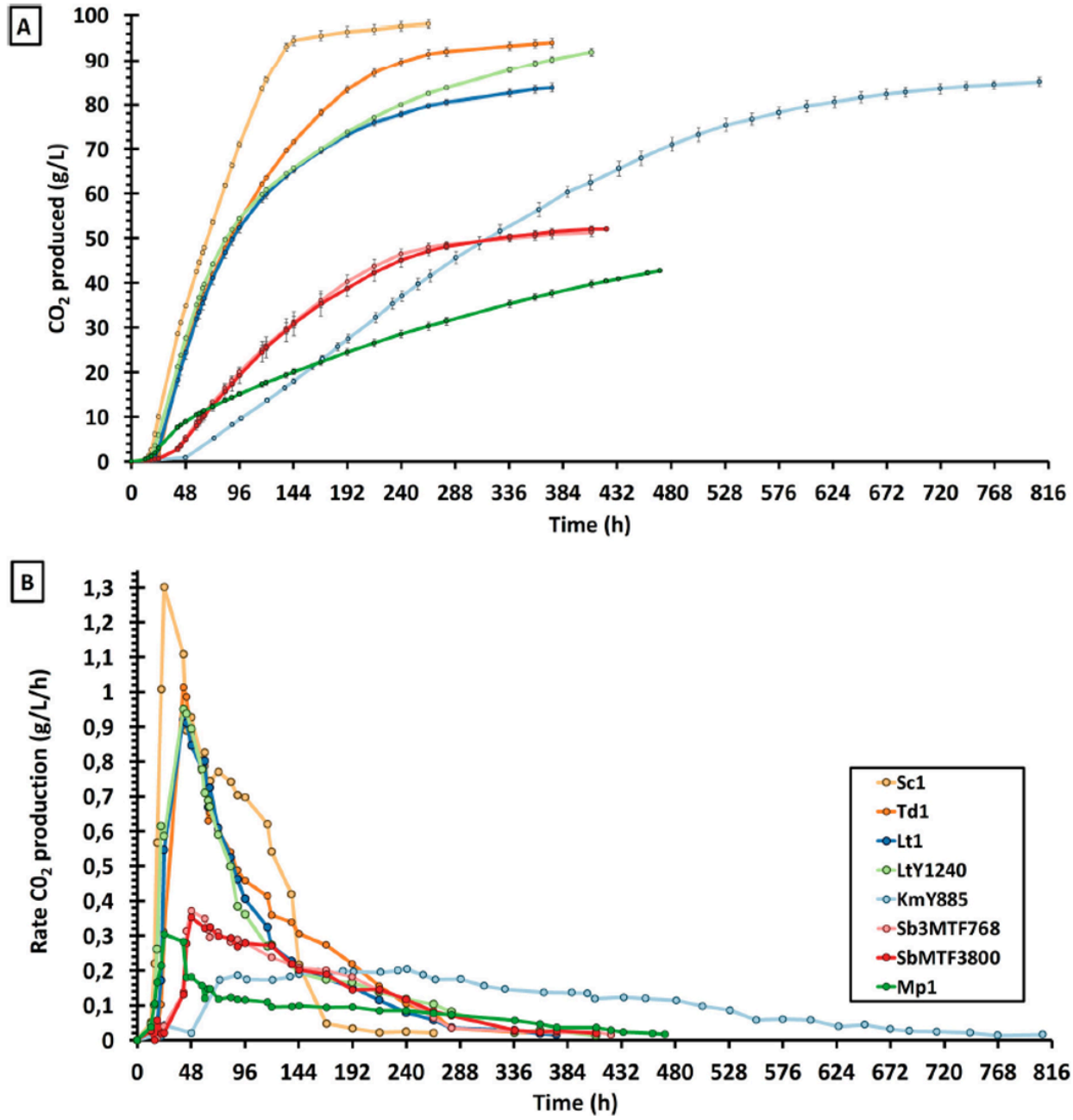


Figure 3: The fermentation kinetics and rate of $CO_2$ production in the eight yeast strains tested.

Take-home message: *S. cerevisiae* ferments to dryness, while non-*Saccharomyces* species do not. Fermentation kinetics vary significantly between species as well as among different strains within the same species. The significant variation in fermentation kinetics between species and among strains within the same species highlights yeast diversity in terms of fermentation kinetics and biomass production under controlled winemaking conditions. While these results were already known for the best part, this first task was essential to establish key time points to consider in the next tasks.

Task 2: The $NAD^+$/NADH and $NADP^+$/NADPH redox cofactors were quantified at each sampling point in each strain. For all strains, the NADP(H) concentrations were observed to be stable in each strain as the fermentation progressed (data not shown). The $NADP^+$/NADPH ratio was also found to be stable at an almost 1:1 ratio in all tested strains. The average concentration was around 8.5 pmol/$10^7$ cells in each strain of *S. cerevisiae*. These results could be explained by the low requirement for this cofactor in a limited set of anabolic reactions (e.g. pentose phosphate pathway, lipid production), and due to the inactivity of the electron transport chain and limited activity of the mitochondria in the absence of oxygen. Similarly, NADP(H) levels showed no significant differences between non-*Saccharomyces* yeasts during fermentation, ranging from 12 to 27 pmol per $10^7$ cells with minimal variation. The $NADP^+$/NADPH ratio remained stable between 0.68 and 1.09, with Lt1, KmY885, and SbMTF3800 slightly favouring NADPH during early growth but balancing out later.

In stark contrast, the total NAD(H) concentrations underwent significant changes in all tested strains. In *S. cerevisiae* (Figure 4), total NAD(H) decreased rapidly after the mid-exponential phase of the yeast growth along with the ratio of $NAD^+$/NADH. However, specific differences were observed between strains regarding the concentrations of total NAD(H) as well as the ratio of $NAD^+$/NADH for all tested strains. This result was unexpected, as it challenged the paradigm and popular assumption that these concentrations and ratios were stable over time. After a thorough review of the literature, it seems that these assumptions were based on the dynamics of NAD(H) cofactor during respiration conditions, which contrast the anaerobic conditions of the wine fermentation. As *S. cerevisiae* is Crabtree positive, it ferments in sugar concentrations exceeding 150 mg/L (although this threshold may be strain-dependent), despite the presence of oxygen. These results clearly showed that NAD(H) is degraded over time. This was linked to the import of nicotinic acid, the original substrate of $NAD^+$, which is discussed further in milestone 2, task 2. In Figure 4, it is evident that the ratio of $NAD^+$/NADH, initially in favour of $NAD^+$, becomes increasingly in favour of NADH as the fermentations progress. Strain-specific differences were observed that were linked to the differences in the production of metabolic end products from central carbon metabolism during fermentation.

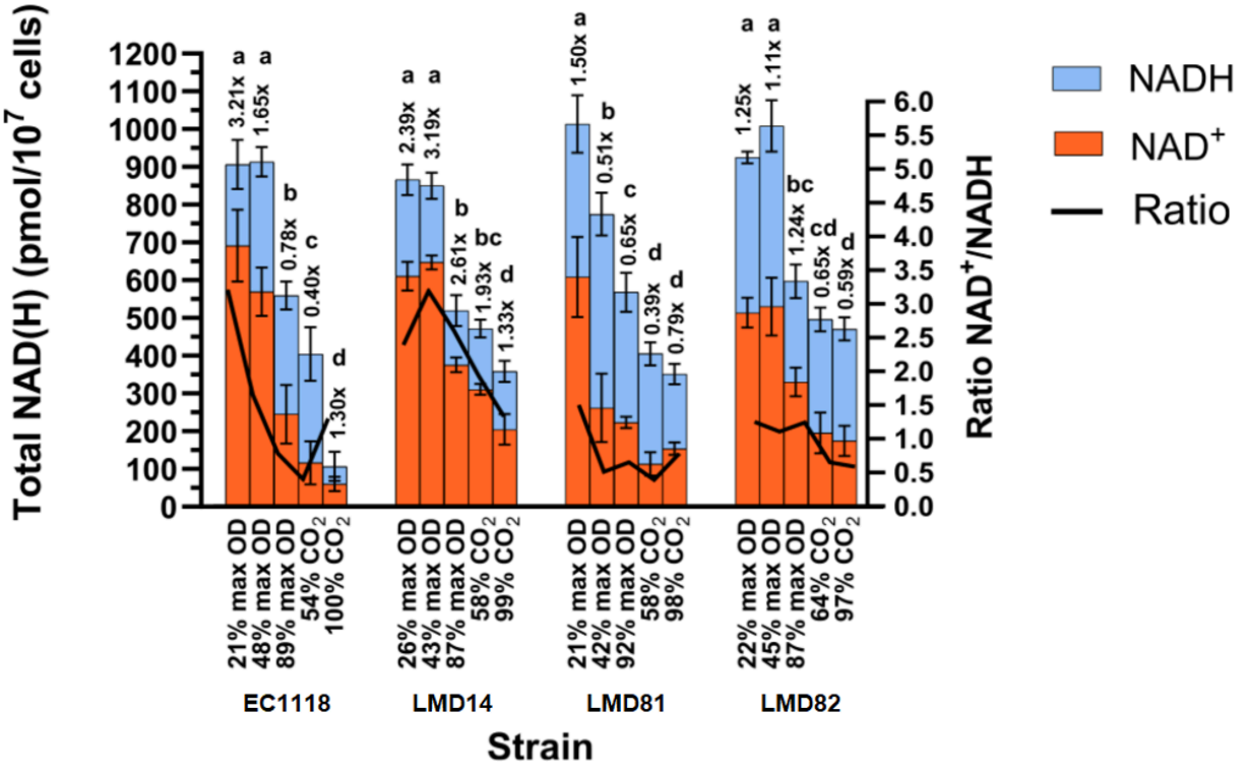


Figure 4: Kinetics of NAD(H) intracellular content in strains EC1118, LMD14, LMD81 and LMD82 during fermentation of synthetic grape juice. The ratio of cofactors is depicted by the black line.

Regarding the other yeast species, total NAD(H) concentrations (Figure 5) varied significantly by species (with smaller differences between strains) and fermentation stage. High-performing strains (Sc1, Td1, LtY1240) maintained NAD(H) levels above 100 pmol/$10^6$ cells until 30 g/L $CO_2$ was released, after which levels dropped sharply. Strains with lower fermentation capacity had lower initial NAD(H) concentrations and experienced declines during late fermentation. The $NAD^+$/NADH ratio varied widely; *L. thermotolerans* showed a lower ratio compared to Sc1 and Td1, while KmY885 maintained a high ratio even after producing substantial $CO_2$. SbMTF3768, SbMTF3800, and Mp1 faced near depletion of $NAD^+$ by 30 g/L $CO_2$. Overall, all strains eventually favoured NADH over $NAD^+$, with varying degrees of reduction. Some yeasts including Sb and Mp could not persist until the 60 g/L sampling point. Interestingly, they revealed a premature and severe redox imbalance in favour of NADH with barely any $NAD^+$ remaining by the 30 g/L $CO_2$ released time point. This is, no doubt, to be correlated to their weak fermentation ability, but this has never been reported before. Indeed, such a severe redox imbalance would induce a collapse of the overall metabolism.

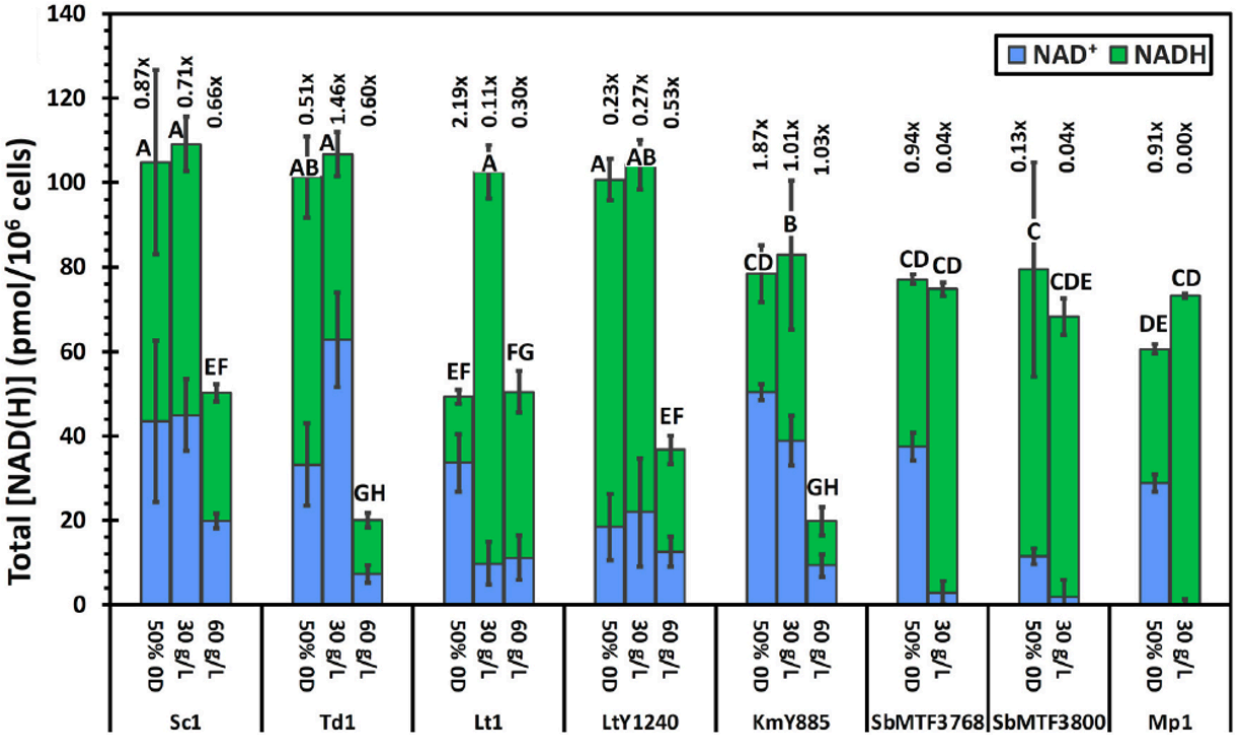


Figure 5: Redox cofactors intracellular levels. A: Total NAD(H) levels in pmol/$10^7$ cells were measured at different physiological phases in wine yeast during fermentation.

Furthermore, the intracellular availability of $NAD^+$ and NADH varied distinctly during fermentation, depending on the yeast species, and these changes in redox status dynamics likely influenced their respective fermentation kinetics and performance. Strains Sc1, Td1, Lt1, and LtY1240 exhibited high NAD(H) content during the initial stages of fermentation, averaging 97 pmol/$10^6$ cells, compared to 72 pmol/$10^6$ cells for other strains, with a $NAD^+$/NADH ratio favouring NADH throughout. These stronger fermenting strains maintained their $NAD^+$ levels by 60 g/L $CO_2$ release, indicating their ability to manage redox balance throughout fermentation.
In contrast, strains Mp1, SbMF3800, and SbMTF3768 showed lower NAD(H) levels and experienced depletion of $NAD^+$ as early as when 30 g/L of $CO_2$ was produced, with concentrations ranging from undetectable levels. These strains displayed poor growth and were unable to complete fermentation. Notably, KmY885 balanced $NAD^+$ and NADH levels with a $NAD^+$/NADH ratio around 0.9, despite having low NAD(H) concentrations. Although KmY885 exhibited weak growth, it consumed most sugars at a slow but steady rate, probably in connection to this low NAD(H) concentration. Overall, the limited ability to regenerate $NAD^+$ appears to contribute to the poor growth and fermentation capacities of certain wine yeast species.

Take-home message: During the course of fermentation, while NADP(H) levels and the $NADP^+$/NADPH ratio remain stable across different yeast strains, NAD(H) concentrations and the $NAD^+$/NADH ratio show significant strain-specific variations. Contrary to previous assumptions, NAD(H) concentrations decline as fermentation progresses, particularly after the mid-exponential phase, with weaker fermenting strains showing more pronounced declines. This shift reflects the accumulation of NADH at the expense of $NAD^+$ as fermentation advances and the carbon source is consumed. Unlike

*S. cerevisiae* and the stronger non-*Saccharomyces* yeasts, weak fermenters possess a lower NAD(H) pool and are not able to sustain the regeneration of $NAD^+$. Subsequently, their populations decline prematurely. The strain-specific variations in NAD(H) concentrations and $NAD^+$/NADH ratios during fermentation highlight the critical role of yeast persistence when selecting yeasts to use in winemaking. Yeasts such as *S. cerevisiae*, which can effectively re-oxidise $NAD^+$ (at least to a certain extent) and maintain metabolic activity, are better suited for complete fermentations, especially under anaerobic conditions. In contrast, weak fermenters struggle to persist, leading to incomplete fermentation.

Task 3: The metabolic end-products produced by all strains investigated in this study were quantified and the results for *S. cerevisiae* EC1118 and the non-*Saccharomyces* yeasts are summarised in Figures 6 and 7. The data related to the other strains of S. cerevisiae are not shown, but they are available upon request. For *S. cerevisiae,* the concentrations of ethanol, glycerol, succinic acid, and acetic acid produced were generally consistent with expectations based on existing knowledge of the strains. However, several observations merit attention. Higher acetic acid production is typically viewed as a compensatory mechanism for yeast to maintain redox balance following higher glycerol production. While this holds for most strains, it was notably not the case for LMD14, which produced high levels of glycerol but low levels of acetic acid. Redox balance in LMD14 was likely achieved through increased succinic acid production. LMD82 produced the lowest glycerol levels, yet its succinate and ethanol levels were similar to those of EC1118 and LMD81. This implies that this strain possesses yet another mechanism to maintain its redox balance.

As anticipated, LMD82 generated significantly less acetate compared to its parental strain LMD81. LMD82 also exhibited lower levels of higher alcohols but produced elevated amounts of butanol, decanoic acid, and ethyl decanoate. Notably, higher $NAD^+$ levels were observed throughout the fermentation in LMD82 compared to LMD81. LMD14 produced high levels of glycerol and succinic acid and low levels of ethanol, which would typically lead to NADH accumulation. Despite this, the redox ratio in LMD14 remained $NAD^+$-favoured throughout fermentation. The decrease in $NAD^+$ over time was gradual and less abrupt in LMD14 compared to the other strains studied. The change in the $NAD^+$/NADH ratio was also less pronounced. This could tentatively be attributed to the increased production of higher alcohols such as butanol and propanol, as well as acetate esters like isoamyl alcohol, and isoamyl acetate. In the Ehrlich pathway, the production of these compounds leads to the regeneration of $NAD^+$ from specific amino acids and sugars. Since acetate esters are derived from acetyl-CoA, this could explain why LMD14 produces less acetic acid, as its precursor is metabolised into acetate esters to maintain redox balance. These results highlight the distinct metabolic strategies of different yeast strains in maintaining redox balance.

Significant differences were observed in the yields of end-products in the non-*Saccharomyces* yeasts, clearly revealing the metabolic fluxes adopted by the different species and strains, in connection with the management of their redox cofactors, especially NAD(H). Glycerol formation is key for regenerating $NAD^+$ from NADH during fermentation. Glycerol yields were inversely correlated with yeast growth

and fermentation capacity. *M. pulcherrima* and the other weakly fermentative species have higher $NAD^+$ regeneration demands. In strains like Td1, Lt1, LtY1240, and KmY885, the glycerol pathway meets $NAD^+$ needs, while in Mp1, SbMTF3768, and SbMTF3800, it does not, leading to fermentation challenges. Lactic acid production in *L. thermotolerans* varied greatly between the two strains investigated, but it did not significantly impact redox balance, but the strain that produced the highest levels of lactic acid also produced the most biomass. Therefore, it could be hypothesised that lactic acid production serves as a redox balance mechanism to compensate the increased biomass. Some variation in succinate were observed among the different yeasts tested.

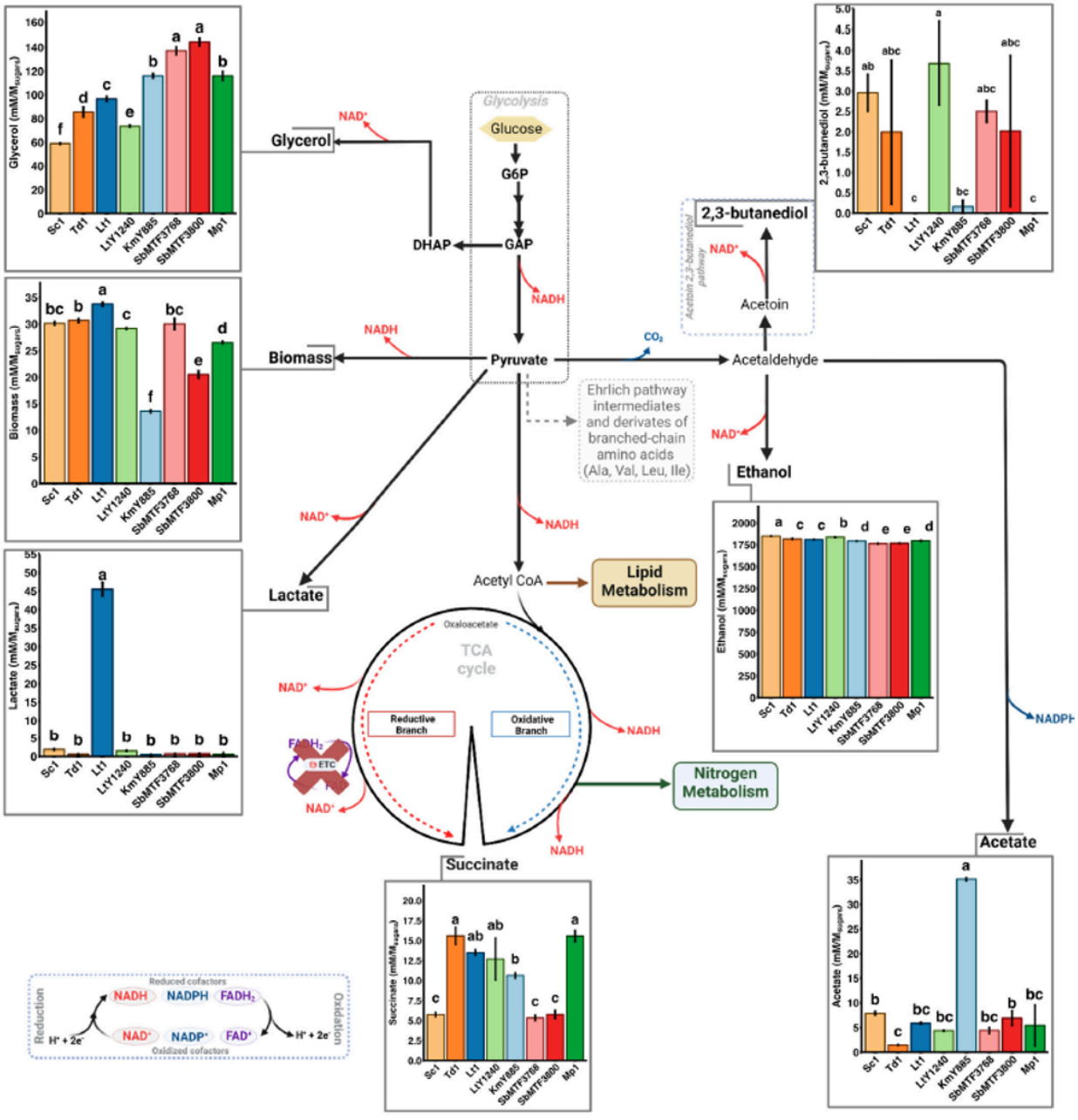


Figure 6: Production yields of the main end-products of central carbon metabolism for each tested yeast.

Clear differences in secondary metabolism were observed amongst yeast species, highlighting significant phenotypic diversity. Monitoring intracellular redox cofactors revealed that these differences are intrinsically linked to how yeasts manage their redox balance. As reported above, yeasts with poor fermentation performance, like *M. pulcherrima* and *S. bacillaris*, struggled to maintain redox balance during anaerobiosis. Stronger fermenters, such as *K. marxianus*, achieved redox balance through varying metabolite concentrations, with higher 2,3-butanediol production compensating for biomass yield and higher glycerol production partially offsetting succinic acid yield.

The impact of redox status on volatile compound production was also evident (Figure 7). Strains with high NADH/$NAD^+$ ratios and low fermentation capacities favoured higher alcohol production over fusel acids due to limited $NAD^+$ availability. *M. pulcherrima* and *S. bacillaris* produced fewer medium-chain fatty acids (MCFAs) and acetate esters but higher levels of isobutanol, reflecting their reduced capacity for oxidised compound production. Conversely, KmY885, which maintained balanced $NAD^+$ and NADH levels, produced more isoamyl alcohol and acetate esters, demonstrating that redox status significantly influences volatile compound formation. Similarly, differences were observed between *S. cerevisiae* strains (data not shown) with different major volatiles linked with differences in redox metabolism, highlighting the different mechanisms that yeasts may use to regenerate redox cofactors.

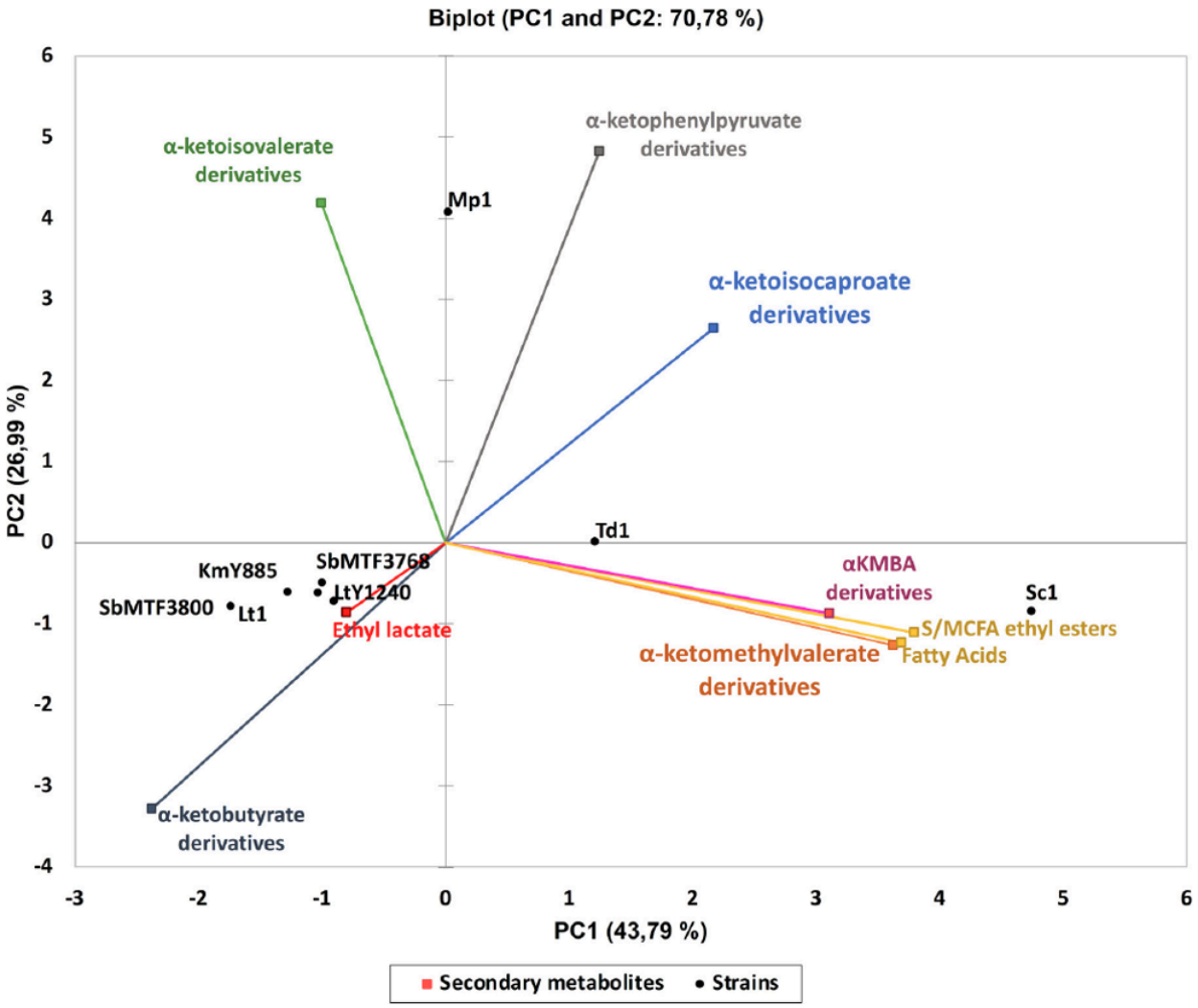


Figure 7: Principal component analysis biplot of the aroma compounds that were quantified at the end of fermentation (analysed according to the intermediates or product of their respective metabolic pathways) and the various strains. Aroma compounds are analysed based on the keto acid precursors involved in the metabolic pathways that produce these compounds, with the exception of MCFAs and their ethyl esters derivatives, which are synthesised from acetyl-CoA metabolism.

Lactic production in *L. thermotolerans* was further investigated as an illustration of the impact of a natural redox perturbation on redox metabolism and ultimately the production of metabolites of oenological interest. The timing of lactic acid production was investigated, together with the influence of selected environmental factors (i.e. oxygen and temperature).

Twelve strains of *L. thermotolerans* and two strains of *L. waltii* were screened for lactic acid production in synthetic grape juice. Only three *L. thermotolerans* strains and the two *L. waltii* strains produced significant levels of lactic acid. This increased lactic acid production was accompanied by a lower ethanol and glycerol production. Like the production of glycerol, lactic acid production coincided with the yeast growth phase. Once again, this clearly highlights its role in redox balancing.

The impact of temperature on lactic acid production was investigated on two *L. thermotolerans* strains and one *L. waltii* strain. Two temperatures were tested: 20°C and

30°C. The production of lactic acid was found to be significantly higher at 20°C than at 30°C for the two *L. thermotolerans* strains, but no significant difference was observed for the *L. waltii* strain.

The impact of oxygen was tested on two strains of *L. thermotolerans*: one lactic acid producer (Lt1) and one non-producer (Y1240) (Figure 8). Semi-aerobic conditions with regular aeration were compared to self-induced anaerobiosis.

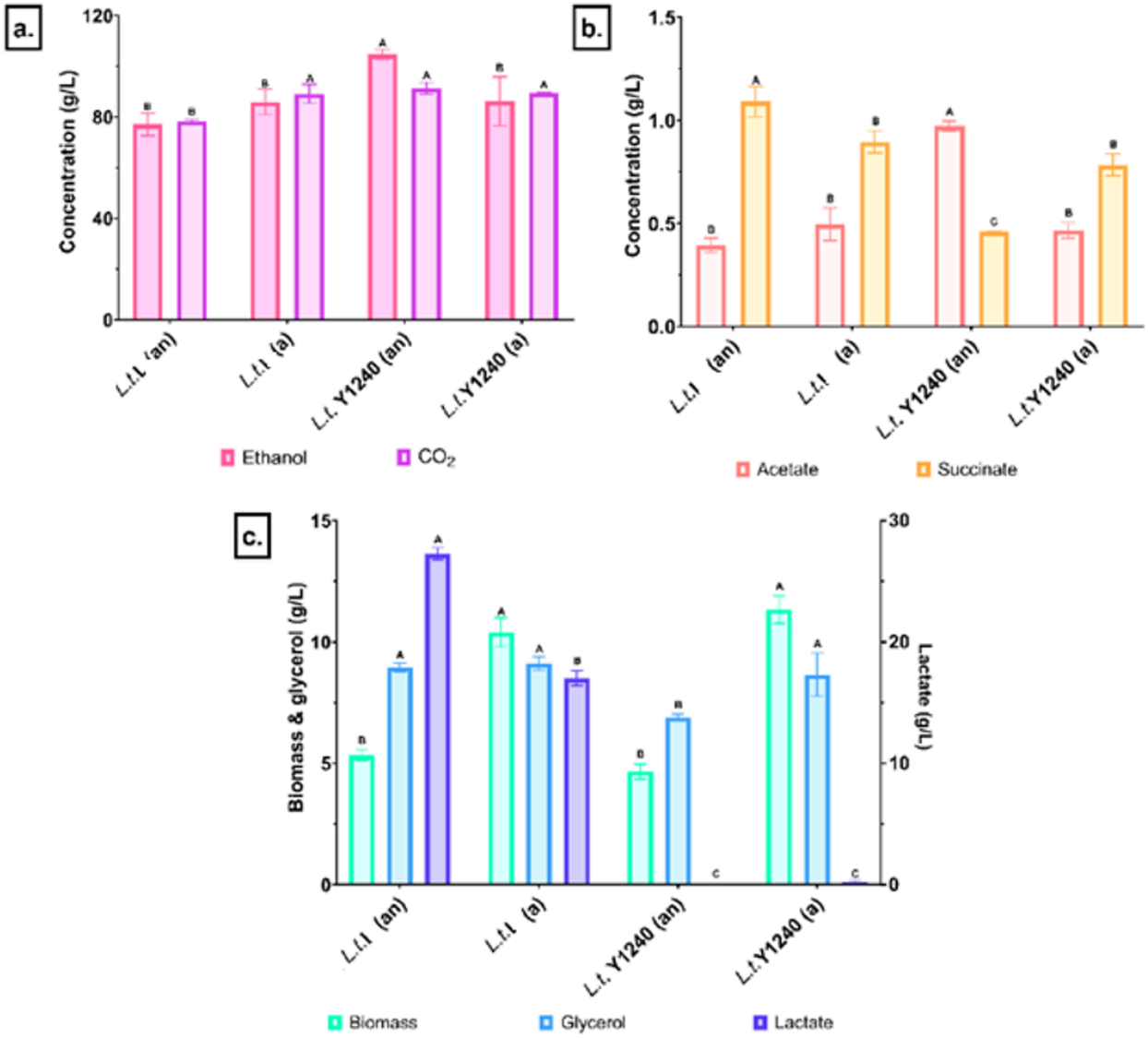


Figure 8: Concentrations of the main primary metabolite and biomass produced by two strains of *L. thermotolerans* – one lactic acid producer (Lt1) and one non-lactic acid producer (Y1240) – in aerated (a) and self-induced anaerobic conditions (an).

The data showed that the presence of oxygen resulted in lower lactic acid production, higher biomass, lower succinic acid, higher acetic acid and unchanged ethanol and glycerol levels in the Lt1-driven fermentations. In contrast, in the fermentations carried out by Y1240, the presence of oxygen resulted in higher biomass, but in glycerol, lower ethanol higher acetic acid, but lower succinic acid. These results clearly demonstrate the interplay between these metabolites as an attempt for the two strains to maintain their redox balance using different metabolic mechanisms.

Take-home message: The metabolic strategies of different yeast strains for managing redox balance during fermentation significantly impact their performance and aroma profiles. For instance, *S. cerevisiae* strains exhibit varied approaches to redox management: LMD14 focuses on succinic acid and higher alcohols production, whereas LMD82 produces less glycerol but maintains similar levels of succinate and ethanol as LMD81. Glycerol production, crucial for regenerating $NAD^+$, inversely correlates with fermentation capacity, with strains like Td1 and Lt1 using this mechanism effectively, while Mp1 does not, thereby impacting its efficiency and overall fermentation performance. Additionally, the redox status influences volatile compound production. Yeasts with high NADH/$NAD^+$ ratios, such as *M. pulcherrima* and *S. bacillaris*, typically generate more higher alcohols and fewer medium-chain fatty acids and acetate esters. Conversely, strains like KmY885, which balance their redox state, produce more desirable aroma compounds. The redox management of each yeast strain, determined by its ability to produce various concentrations of metabolic end-products and its intracellular pool of NAD(H), determines the aromas produced during fermentation, and by knowing this, winemakers can predict and exploit the resulting aroma profiles to enhance desirable wine characteristics. Lactic acid production varies among *L. thermotolerans* strains with high lactic acid producers like Lt1 substituting ethanol or lactate production without significantly affecting the redox balance. Oxygen was shown to impact lactic acid producers and non-producers differently. Lactic acid production in *L. thermotolerans*, together with oxygen exposure, represent interesting features could serve to alter metabolic footprint and possibly lower the alcohol content of wine. Nevertheless, it should be further investigated.

**Milestone 2:**

Task 1: A multifactorial experiment was conducted to evaluate the effects of tryptophan, thiamine, and nicotinic acid on fermentation using a Box-Behnken statistical design on each tested strain. These three factors were selected as they can potentially impact redox metabolism. Indeed tryptophan and nicotinic acid are precursors or NAD(P) and thiamine is a cofactor of several enzymatic reactions within the broader alcoholic fermentation metabolism. Each factor was tested at limiting, average, and high concentrations typically found in grape must. The fermentation kinetics of the four *S. cerevisiae* strains were monitored through weight loss, with dry biomass measured at the end of fermentation. Although all treatments fermented to dryness, those with limited nicotinic acid concentrations exhibited the slowest fermentation rates. Indeed, nicotinic acid was the most significant contributor to the variance in fermentation duration across all four strains (Figure 9), accounting for over 50% of the observed differences, followed by thiamine. In contrast, tryptophan had a negligible impact on fermentation duration but significantly influenced biomass production, likely due to its role as a preferred nitrogen source rather than as an $NAD^+$ precursor, particularly in the absence of oxygen. No consistent trends were observed for the production of primary metabolites (data not shown).

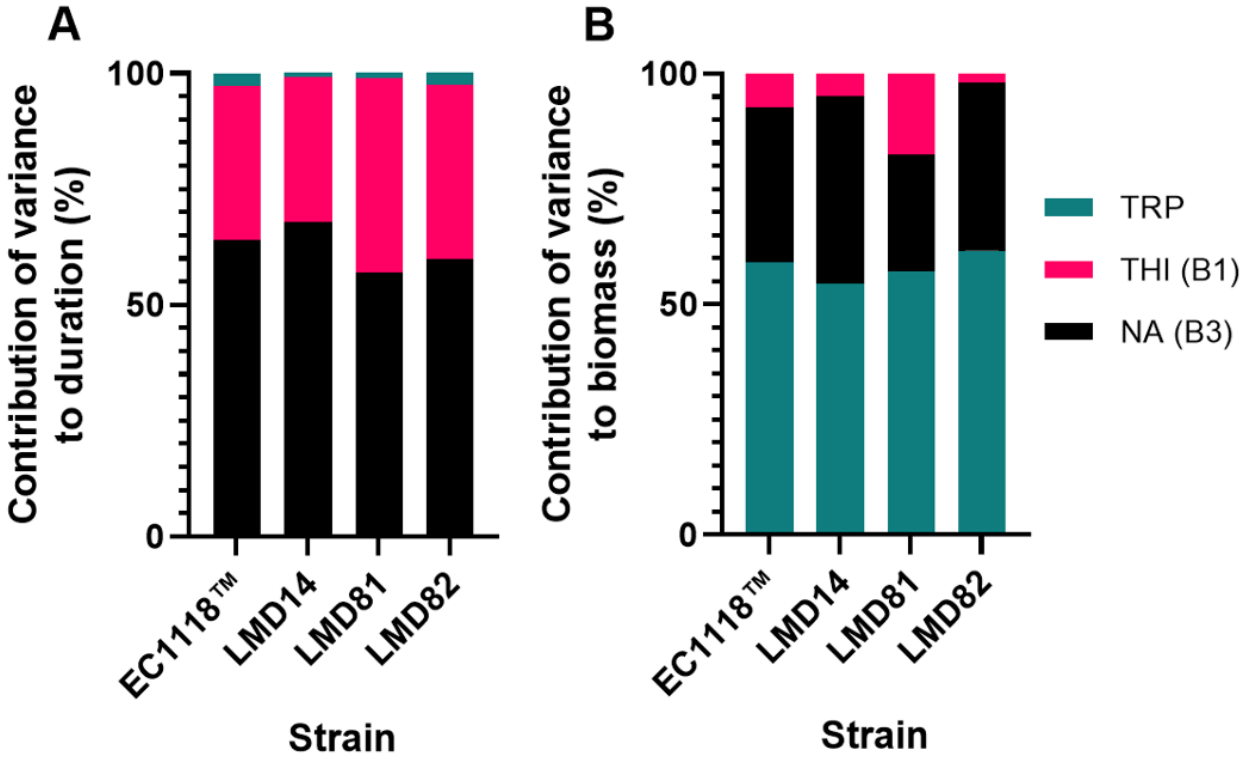


Figure 9: The contributions of tryptophan (TRP), vitamin B1 in the form of thiamine HCl (THI) and vitamin B3 as nicotinic acid (NA) to the variance observed in fermentation duration (A) and biomass (B) for each strain.

Similarly in the tested non-*Saccharomyces* yeast strains, nicotinic acid and, to a lesser extent, thiamine significantly influenced fermentation kinetics, and metabolite production, while tryptophan's effects were minimal. Nicotinic acid emerged as the only factor consistently affecting the fermentation rate across all strains except SbMTF3800 (Figure 10). When nicotinic acid was limited, yeasts including Lt1, LtY1240, and KmY885 showed a substantial decrease in intracellular NAD(H) and NADP(H) pools. Despite this, growth potential was not hindered, suggesting that even low cofactor levels were sufficient for their electron carrier role. Compared to thiamine, nicotinic acid deficiency had a more pronounced effect on yeast activity, leading to a significant decrease in intracellular NAD(H) pools (with the notable exception of *S. bacillaris*). This limited availability of NAD(H) cofactors caused a general slowdown in fermentation rates and, in all species except *S. cerevisiae*, incomplete sugar consumption. Due to the critical role of nicotinic acid as a direct precursor of $NAD^+$, Task 2 was designed to focus further on its metabolism.

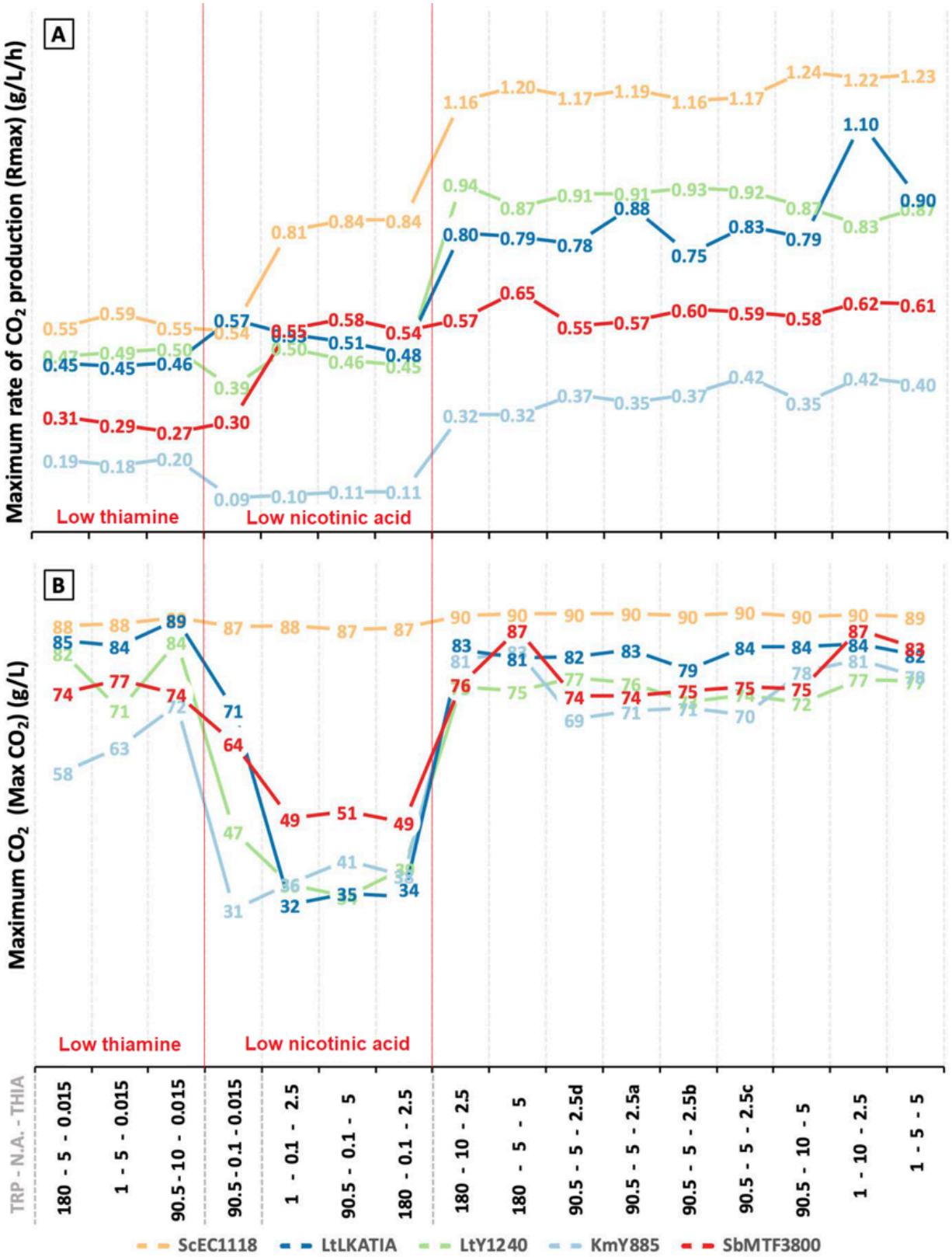


Figure 10. The maximum rate of $CO_2$ production and the maximum production of $CO_2$ in selected yeast species: *S. cerevisiae* (EC1118), *L. thermotolerans* (Lt1 and Y1240) and *K. marxianus* (Y885).

Take-home message: Nicotinic acid plays a crucial role in fermentation, and a deficiency

in this vitamin significantly impacts the fermentation rate and redox balance across most yeast species. Limiting levels of nicotinic acid impact redox metabolism and lead to slower fermentation, which can affect the completion of sugar consumption causing stuck fermentations. Additionally, low levels of thiamine negatively impacted the fermentation rate in all tested strains (as previously reported in *S. cerevisiae*), except for *S. bacillaris*. Therefore, ensuring adequate nicotinic acid and thiamine levels is essential for optimal fermentation performance, particularly in non-*Saccharomyces* species.

Task 2: Fermentations were conducted in synthetic grape juice containing three levels of nicotinic acid (0.1, 2.0 or 4.0 mg/L as in Task 1) with only EC1118 as a representative strain. Samples were taken at: 1 h, 7 h, 13 h, 24 h, mid-exponential phase, entry into stationary phase (corresponding to 30% of fermentation completion) and at 60% of fermentation completion). Fermentation kinetics and yeast population dynamics are shown in Figure 11. The 2 mg/L and 4 mg/L nicotinic acid treatments exhibited similar and expected fermentation kinetic patterns, whereas the 0.1 mg/L treatment resulted in low biomass formation and sluggish fermentation, though dryness was eventually reached 24 h after the other treatments.

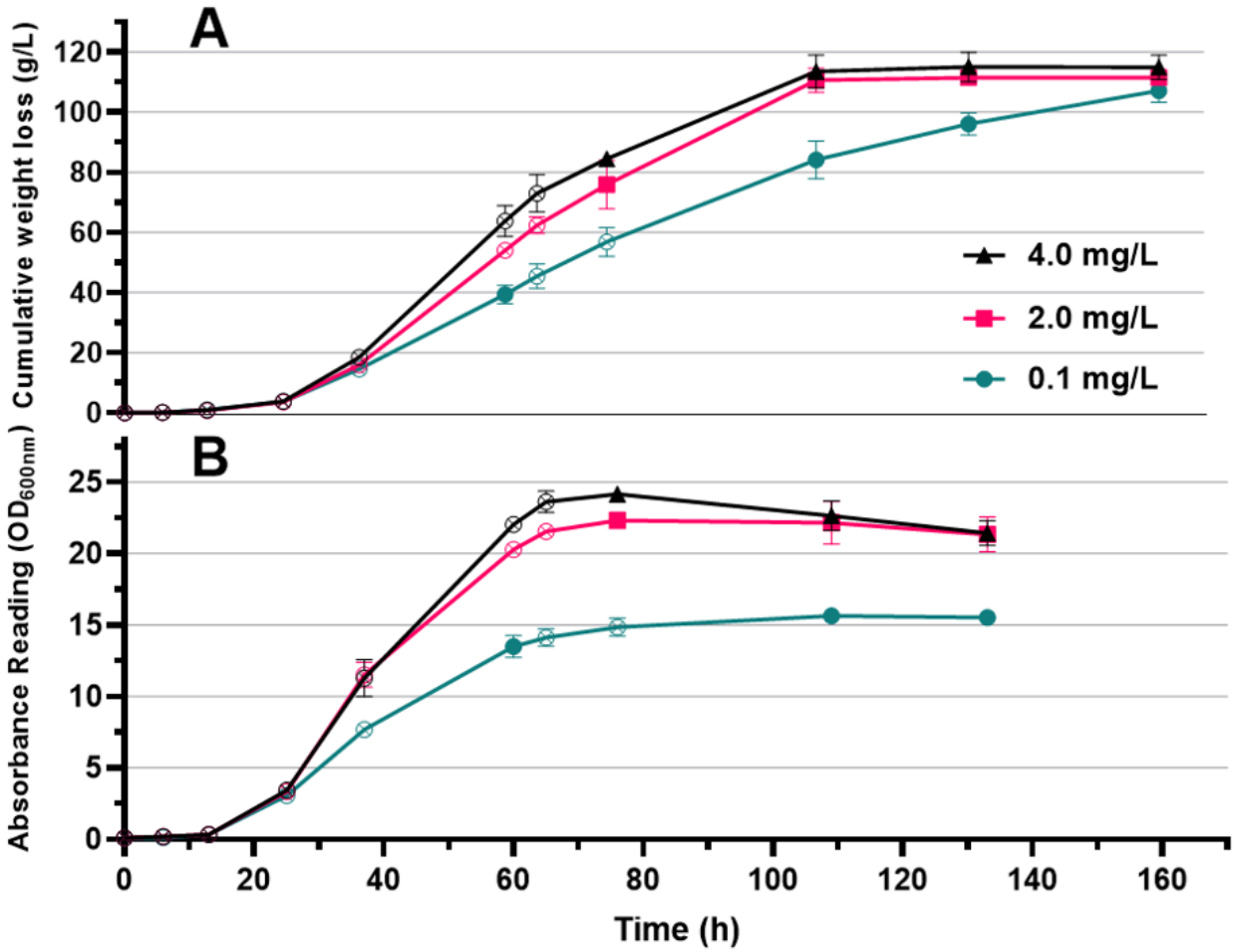


Figure 11: The cumulative weight loss (A) and yeast growth (B) for each nicotinic acid treatment.

NAD(H) levels were quantified intracellularly at each sampling point (Figure 12). The results show similar trends to those shown in Figure 4: the concentration of NAD(H) decreased over time after the 24 h time point (corresponding approximately to 25% total biomass produced) with NADH becoming more and more dominant over $NAD^+$ because the reduction of $NAD^+$ was not matched by a continuous reoxidation of NADH at the same rate. In the 0.1 mg/L nicotinic acid treatment, the trend was even more acute with the decrease in NAD(H) starting earlier (between 13 and 24 h) and the cell experiencing a severe redox imbalance in favour of NADH. The clear imbalance in the $NAD^+$/NADH ratio in the 0.1 mg/L treatment is likely the cause of the low biomass produced and the resulting sluggish fermentation observed.

The metabolic fate of nicotinic acid was determined in Figure 13, with three distinct phases proposed. The first phase occurs immediately after inoculation and is followed by an accumulation phase of nicotinic acid. The final phase is defined by the subsequent recycling of redox cofactors and export of vitamin B3 (Figure 12). The inoculation phase consists of the first 24 h as the yeast slowly increases its metabolism and imports nicotinic acid for redox cofactor metabolism. In the accumulation phase, the cells have consumed all nicotinic acid. A general vitamin B3 uptake microbiological assay determined that all extracellular vitamin B3 was assimilated before reaching 50% of maximum yeast growth. This point coincided with peak total NAD(H) levels within the cell as fermentation rates reach their maximum. Thereafter, the recycling phase consists of the export of vitamin B3 in proportion to the initial supplementation level, indicating a threshold for vitamin B3 uptake, beyond which additional supplementation may not enhance fermentation dynamics.

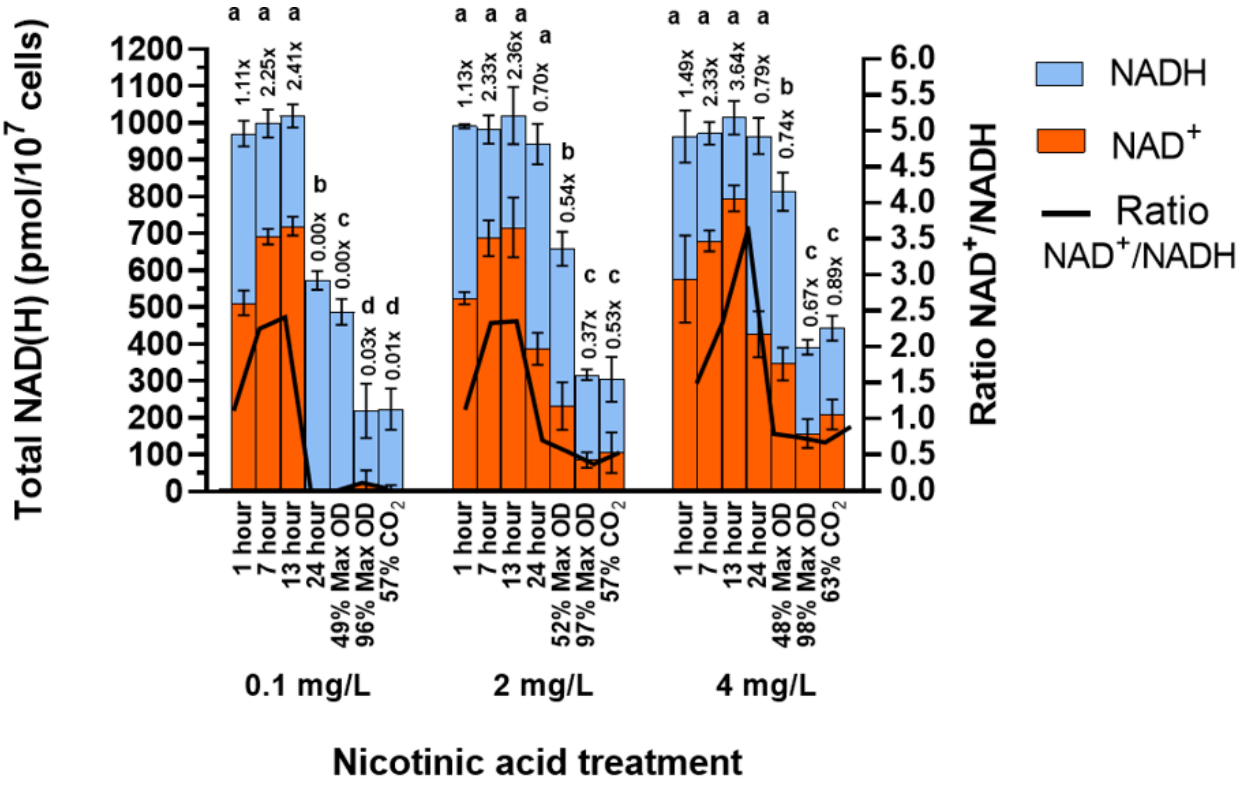


Figure 12: The total levels and ratio of NAD(H) cofactors at each sampling point. The

total NAD(H) level represents the combined concentrations of $NAD^+$ and NADH, while the ratio of cofactors is depicted by the black line

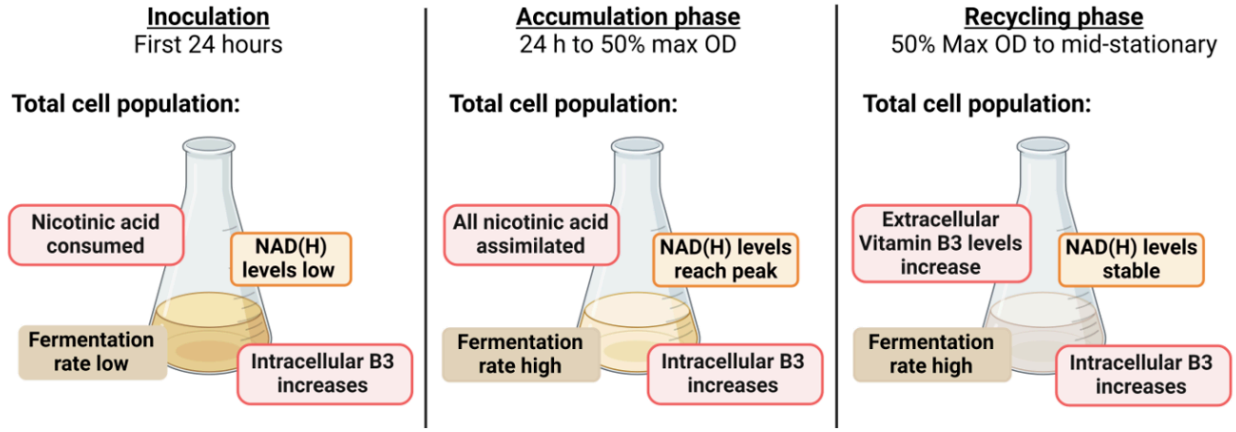


Figure 13: Evolution of nicotinic acid uptake.

Finally, the concentrations of primary metabolites produced in the three treatments were quantified. Similar yields of primary metabolites were observed for the 2 mg/L and 4 mg/L treatments. However, low levels of nicotinic acid were associated with increased production of glycerol, malic acid and acetoin as a mechanism for $NAD^+$ regeneration, a decrease in acetic acid due to acetaldehyde accumulation, and an increase in succinic acid production. From these experiments, it is evident that deficient vitamin B3 causes severe redox imbalance resulting in slower kinetics, and growth and impacting the production of metabolites.

Take-home message: Lower nicotinic acid levels in fermentation cause sluggish kinetics, reduced biomass, and a severe redox imbalance, with NADH dominating over $NAD^+$ as no precursors are available to produce more $NAD^+$ and too few reactions can regenerate $NAD^+$ from NADH. This deficiency also led to altered metabolite production, including increased glycerol, malic acid, and acetoin, as well as decreased acetic acid. These results highlight the importance of adequate vitamin B3 levels to maintain balanced redox status, efficient fermentation, and optimal metabolite profiles. Although not tested, we can hypothesise that with a sequential inoculation with non-*Saccharomyces*, such a scenario (i.e. vitamin B3 deficiency) would likely result in a stuck fermentation and strongly altered metabolic footprint.

Task 3: Since oxygen has strong impact on redox metabolism, fermentations were performed with EC1118 in synthetic grape juice, under strictly anaerobic conditions (below 0.1 mg $O_2$/L) and aerobic conditions where oxygen was continuously provided to the medium (6.4-11.0 mg $O_2$/L). Samples were taken for redox cofactor analysis for both treatments. For the aerobic fermentation experiment, sampling took place 6 h after inoculation as well as 25%, 50%, 75%, and 100% of the maximum yeast growth. For anaerobic fermentations, sampling took place after 6 h, at 25%, 50% 100% of maximum yeast growth as well as 60% and 100% $CO_2$ release. A transcriptomic analysis was performed using RNA sequencing to evaluate the transcription level of genes involved in nicotinic acid uptake and NAD metabolism over time and under

anaerobiosis/aerobiosis in EC1118. Sampling for the RNAseq analysis took place at 6 h, 25%, and 50% maximum growth in both the anaerobic and aerobic experiments.

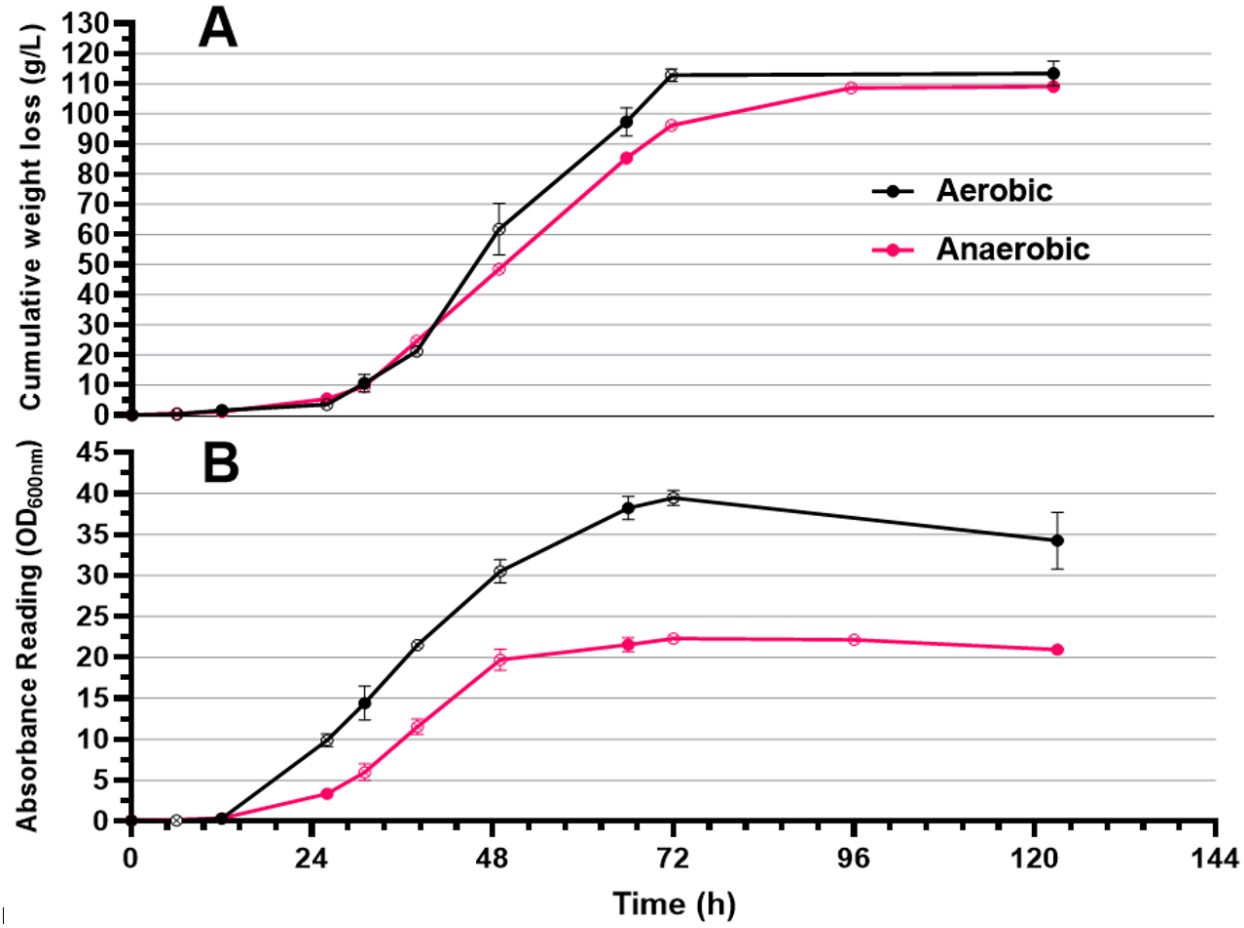


Figure 14: The cumulative weight loss during aerobic and anaerobic fermentation was measured (A) as well as the absorbance (OD600nm) as a proxy for yeast growth (B).

The introduction of oxygen into the medium resulted in significantly faster fermentation kinetics (Figure 14) compared to anaerobic fermentation, which took longer to achieve complete fermentation. Additionally, the presence of oxygen led to significantly higher yeast growth during the fermentation, with this treatment recording 86% more dry biomass compared to the anaerobic treatment. This likely resulted from the efficient use of carbon sources as well as the additional use of proline as a nitrogen source. Unlike in anaerobiosis, where proline cannot be catabolised by *S. cerevisiae*, the presence of oxygen allows for its assimilation when more favourable nitrogen sources are depleted. Notable proline utilisation genes were notably upregulated at 50% max OD under aerobic conditions, after the depletion of more readily assimilable nitrogen sources.

Table 1: Yields for sugar consumed between aerobic and anaerobic treatments.

| Yields: | Anaerobic | Aerobic |
|---|---|---|
| EtOH (g/g sugar consumed) | 0.46 | 0.43 |
| Acetate (g/g sugar consumed) | 0.0017 | 0.0024 |
| Glycerol (g/g sugar consumed) | 0.028 | 0.07 |
| Glycerol/Ethanol ratio (%) | 6.11 | 16.4 |

The anaerobic treatment reached its maximum OD after 48 h, while the aerobic fermentation reached its maximum OD when the fermentation had already concluded after 72 h. Indeed, no stationary phase was observed in the aerobic treatment as growth continued until the fermentation was completed.

The aerobic treatment led to a notably higher production of glycerol, succinic acid, malic acid, and acetic acid, whereas the anaerobic treatment resulted in elevated levels of ethanol. These outcomes were also reflected in the production yields (Table 1), with the anaerobic treatment showing a higher yield of ethanol, while the aerobic treatment exhibited a higher yield of glycerol and acetic acid. There were no significant differences in the production of carbon dioxide or the level of consumed sugars among the treatments.

Similar to previous results, the $NADP^+$/NADPH ratio was also about 1:1 in both aerobic and anaerobic treatments, however, the levels of each cofactor were approximately three times higher in the aerobic treatment compared to the anaerobic treatment (Figure 15) likely due to the increased requirement for lipid biosynthesis. The total level of NADP(H) remained stable at around 26.66 pmol/$10^7$ cells in the aerobic treatment (Figure 15A) and 8.32 pmol/$10^7$ cells in the anaerobic treatment (Figure 14B). There were no significant differences in the total NADP(H) levels between each sampling point in each treatment.

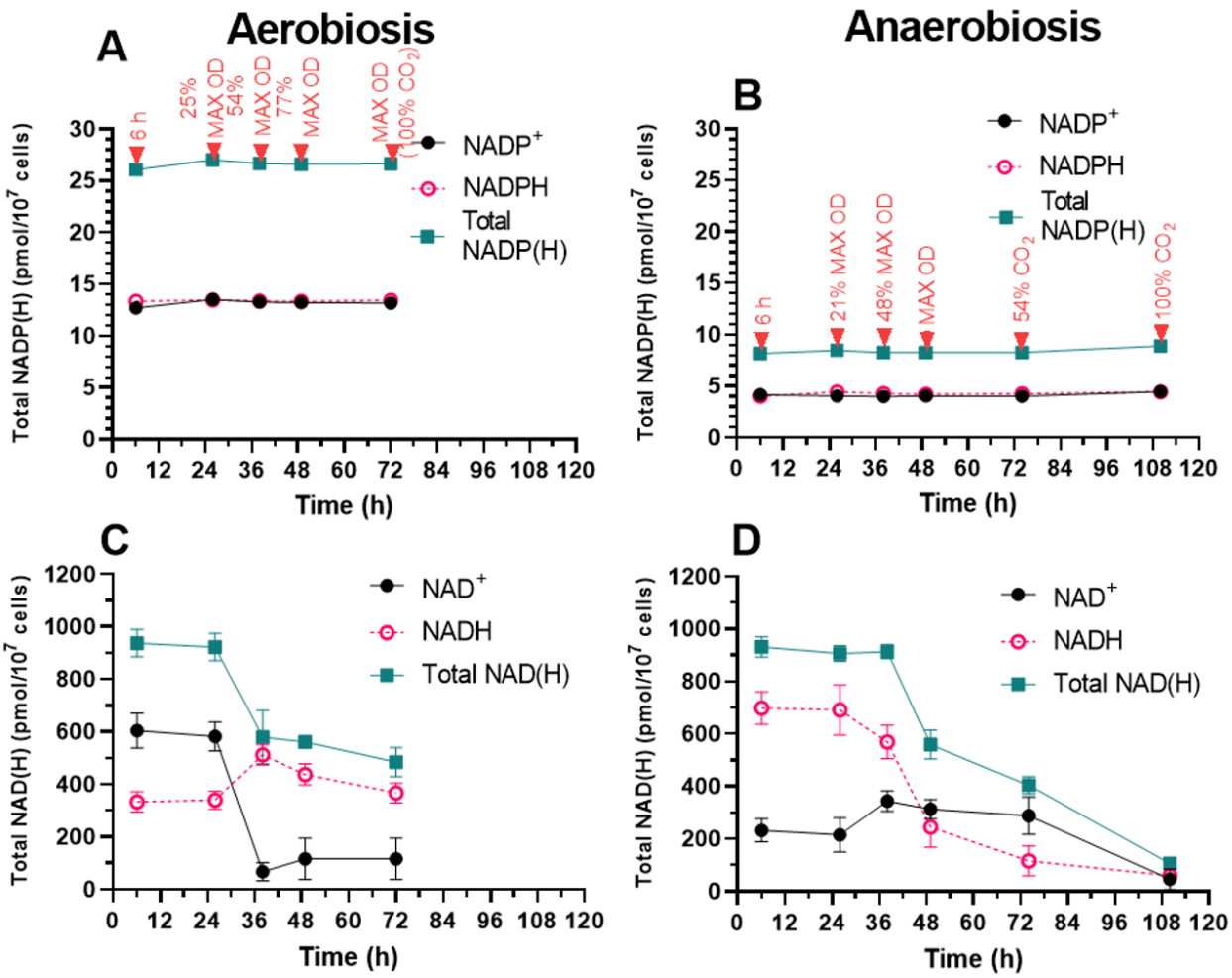


Figure 15. The evolution of each redox factor was investigated. Specifically, $NADP^+$, NADPH, and total NADP(H) levels were determined under aerobic conditions (A) and anaerobic conditions (B). Similarly, $NAD^+$, NADH, and total NAD(H) levels were measured under aerobic conditions (C) and anaerobic conditions (D). All sampling points presented here represent the mean values obtained from triplicate experiments, and the error bars represent the standard deviation between triplicates. Red triangles have been used to label the corresponding physiological phase at each sampling point.
In the aerobic treatment, $NAD^+$ represented the predominant form at the beginning of fermentation– a reversal from the ratio observed in anaerobic conditions where NADH predominated. At the beginning of fermentation, 6 h after inoculation, the total NAD(H) concentrations in the cells in both the anaerobic and aerobic treatments were initially similar at about 906.78-937.53 pmol/$10^7$ cells. However, a decrease to 484.17 pmol/$10^7$ cells and 106.97 pmol/$10^7$ cells was observed in the aerobic and anaerobic treatments, respectively.

In the aerobic treatment, a decrease in total NAD(H) was observed after the 25% max OD point, with no significant changes in redox cofactor levels until the end of fermentation. In line with previous experiments, the anaerobic treatment showed a decrease in total NAD(H) only after the 50% max OD point, with a further decline continuing until the end of fermentation, despite no increase in cell biomass after 48 h. During this period, the NADH/$NAD^+$ ratio increased, and while NADH was initially

dominant, $NAD^+$ became more prevalent after the decline, though the shift was less pronounced than in the aerobic treatment. The transcriptomes of both aerobic and anaerobic samples were compared to identify genes exhibiting significant differential expression at each sampling point and treatment.

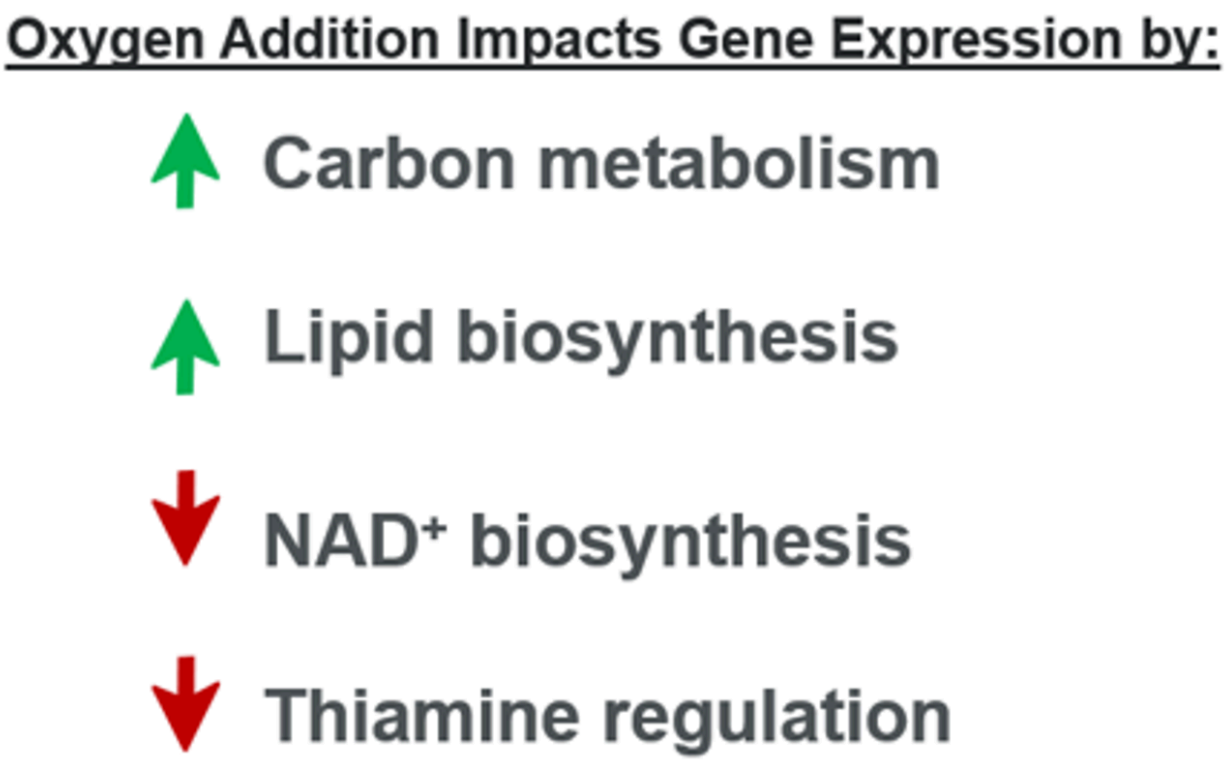


Figure 16. Main metabolic pathways impacted by the addition of oxygen

The gene expression genetic clusters (Figure 16) for carbon metabolism, lipid biosynthesis, and ergosterol biosynthesis showed positive trends, with most genes being upregulated after 6 h under anaerobic conditions. Thiamine metabolism showed a strong, negative trend under anaerobic conditions, with decreased gene expression observed from 6 h onwards. Surprisingly for $NAD^+$ biosynthesis, some genes were differentially expressed, but the overall trend remained neutral, showing both positive and negative profiles.

Under anaerobic conditions, cellular resources are directed towards nicotinamide production, likely due to the specific metabolic needs of anaerobiosis. Genes involved in the first steps of $NAD^+$ *de novo* synthesis exhibited reduced expression at 6 h and 50% max OD, reflecting the oxygen dependence of these reactions. Elevated expression of $NAD^+$ biosynthesis genes under aerobic conditions likely supported the increased biomass observed. The increased expression of thiamine-related genes aligned with the heightened metabolic activity and biomass production driven by oxygen availability after 6 h, consistent with the increased $NAD^+$ demand during aerobiosis observed. Additionally, thiamine may act as a protective agent against stress during aerobiosis, likely due to its role in mitigating reactive oxygen species (ROS). As expected, the vitamin B3 salvage pathway emerged as the preferred route for nicotinamide production from nicotinamide riboside during anaerobiosis. *De novo* $NAD^+$ pathway upregulated during aerobiosis while vitamin B3 salvage pathways are upregulated during

anaerobiosis.

Take-home message: Oxygen availability significantly influences fermentation kinetics, yeast growth, and metabolic profiles in *S. cerevisiae*. Aerobic conditions accelerate fermentation, enhance yeast biomass, and lead to higher production of glycerol, succinic acid, and acetic acid (although remaining under its sensory threshold), while anaerobic conditions favour higher ethanol yields. There are distinct redox cofactor dynamics and gene expression patterns between aerobic and anaerobic fermentations, with aerobic conditions promoting genes involved in carbon metabolism and lipid biosynthesis, and anaerobic conditions promoting the expression of genes involved in $NAD^+$ biosynthesis and thiamine regulation. While the treatment tested in this study (i.e. continuous full aerobiosis) would not be desirable in industry, the control of oxygen exposure could be a strategy to modulate yeast growth, fermentation speed, and the production of key metabolites such as ethanol, glycerol and acetic acid, which directly influence wine quality and style. Strains that are optimised for performance under certain oxygen conditions could be developed to enhance specific wine characteristics. Although this was not tested in this task, this may be particularly relevant for non-*Saccharomyces* yeasts to enhance their contributions.

Task 4: As nicotinic acid and thiamine induced the most extreme behaviours, these treatments were applied to a new set of fermentations in Chenin Blanc grape juice. This was tested in two strains namely EC1118 and LMD14. Fermentation kinetics, primary metabolites and the production of volatile aroma compounds were quantified to validate the results obtained in previous experiments in synthetic grape juice. In a separate fermentation, nicotinic acid was also supplemented at the start of fermentation as well as added at the time when the strains reached their maximum population to assess whether this had an impact on NAD management. Additionally, sensory evaluation was performed at the end of the fermentation to determine the drivers of the sensory differences and the consumers' preferences.

Chenin Blanc juice with the following parameters was used: 23.5°B, 3.65 pH, 5.92 g/L TA, 115 NTUs and 272 mg N/L YAN. Active dry yeasts were inoculated according to packaging instructions. Table 2 shows these treatments.

Table 2: The treatments for Chenin Blanc fermentations. For the THI (B1) treatments, 0.6 mg/L thiamine HCl was supplemented at the start of fermentation while for the $O_2$ addition, 3 mg/L $O_2$ was added per day until 100% Max OD.

| Treatment: | THI (B1) supplement | No THI (B1) supplement | $O_2$ addition | No $O_2$ addition |
|---|---|---|---|---|
| 1 EC | X | | | X |
| 2 EC | | X | | X |
| 3 EC | X | | X | |
| 4 EC | | X | X | |
| 5 LMD14 | X | | | X |
| 6 LMD14 | | X | | X |
| 7 LMD14 | X | | X | |
| 8 LMD14 | | X | X | |

While both EC1118 and LMD14 reached similar maximum OD levels (Figure 17), it is clear that EC1118 fermented slightly faster than LMD14 with the $O_2$ supplement treatments increasing the fermentation rate even further. The addition of $O_2$ did not seem to increase the fermentation rate of LMD14 significantly. The addition of thiamine had no significant impact on the production of biomass or of fermentation kinetics.

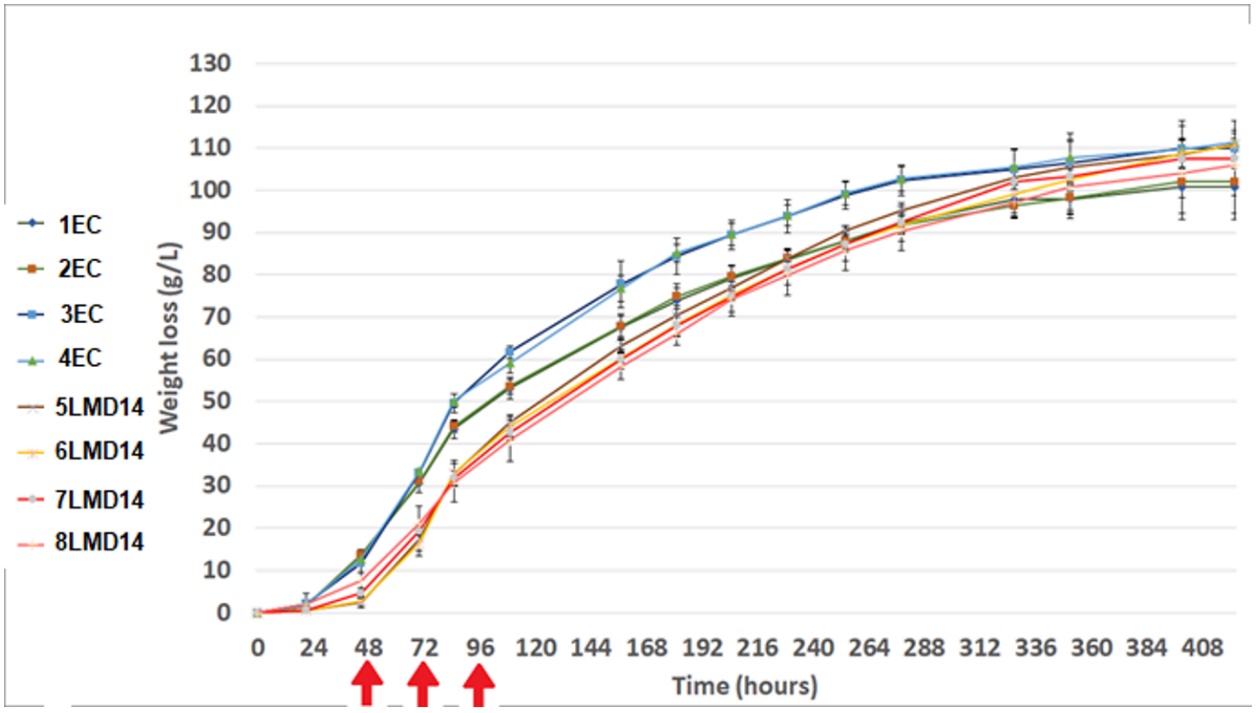


Figure 17: The cumulative weight loss for each treatment was measured. Red arrows denote $O_2$ addition.

Statistical analysis using a logistic model showed that EC1118 reached the mid-exponential phase faster than LMD14. However, no significant differences were found between treatments regarding the time to reach the mid-exponential phase.
Figure 18 summarises the effects of oxygen for EC1118. Indeed, while the thiamine treatments had no significant effects, the impact of oxygen was clear. The addition of oxygen led to increased biomass and a reduced ethanol yield, with a notable rise in propanol due to the higher nitrogen demand under aerobic conditions. Major volatiles

were quantified with significant differences observed between anaerobic and aerobic treatments. Oxygenation increased biomass and glycerol. However, the higher biomass required more lipids, resulting in a substantial decrease in medium-chain fatty acids (MCFAs), compounds that can contribute to undesirable aromas. The Ehrlich pathway showed increased flux, leading to enhanced higher alcohol and ethyl ester . Acetic acid and ethyl acetate also increased slightly, but remained at levels that contributed pleasant fruity notes and were well below legal limits for volatile acidity.
Interestingly, some of the trends highlighted above were opposite in LMD14, thereby highlighting once again the different metabolic behaviour of the latter strain.

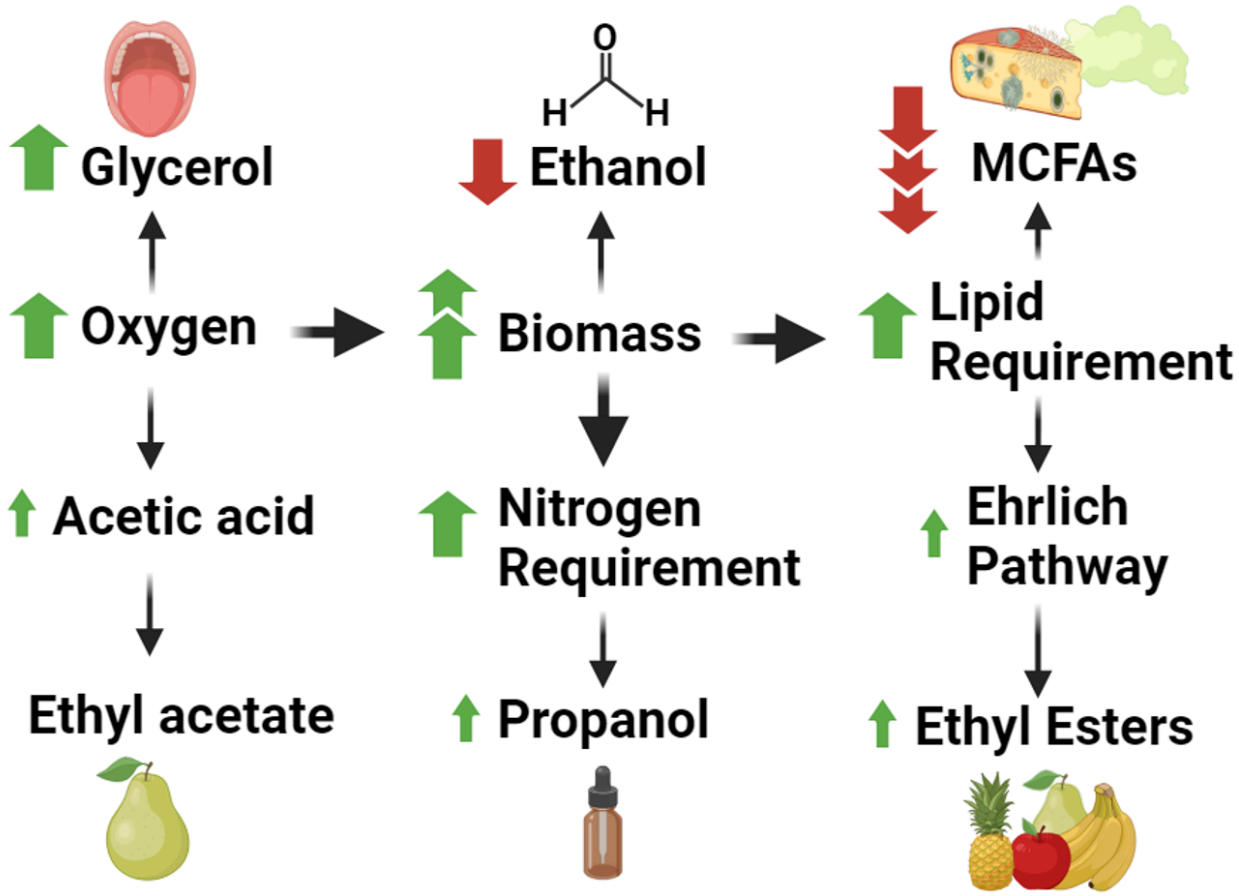


Figure 18: The impact of oxygen addition on major metabolic compounds in the wine obtained from EC1118. Small arrows depict a lower impact whereas bigger arrows depict more of an impact.

The absorbance of the wine at 420 nm was quantified to determine whether oxidation has occurred in the wine (Figure 19). The absorbance in all treatments were below 0.1, which is well below the recognised level of fully oxidised ($OD_{420nm}$ of 0.5). This can also be seen in the images of the wines. Interestingly, the addition of $O_2$ did increase the absorbance readings in EC1118, but not in LMD14.

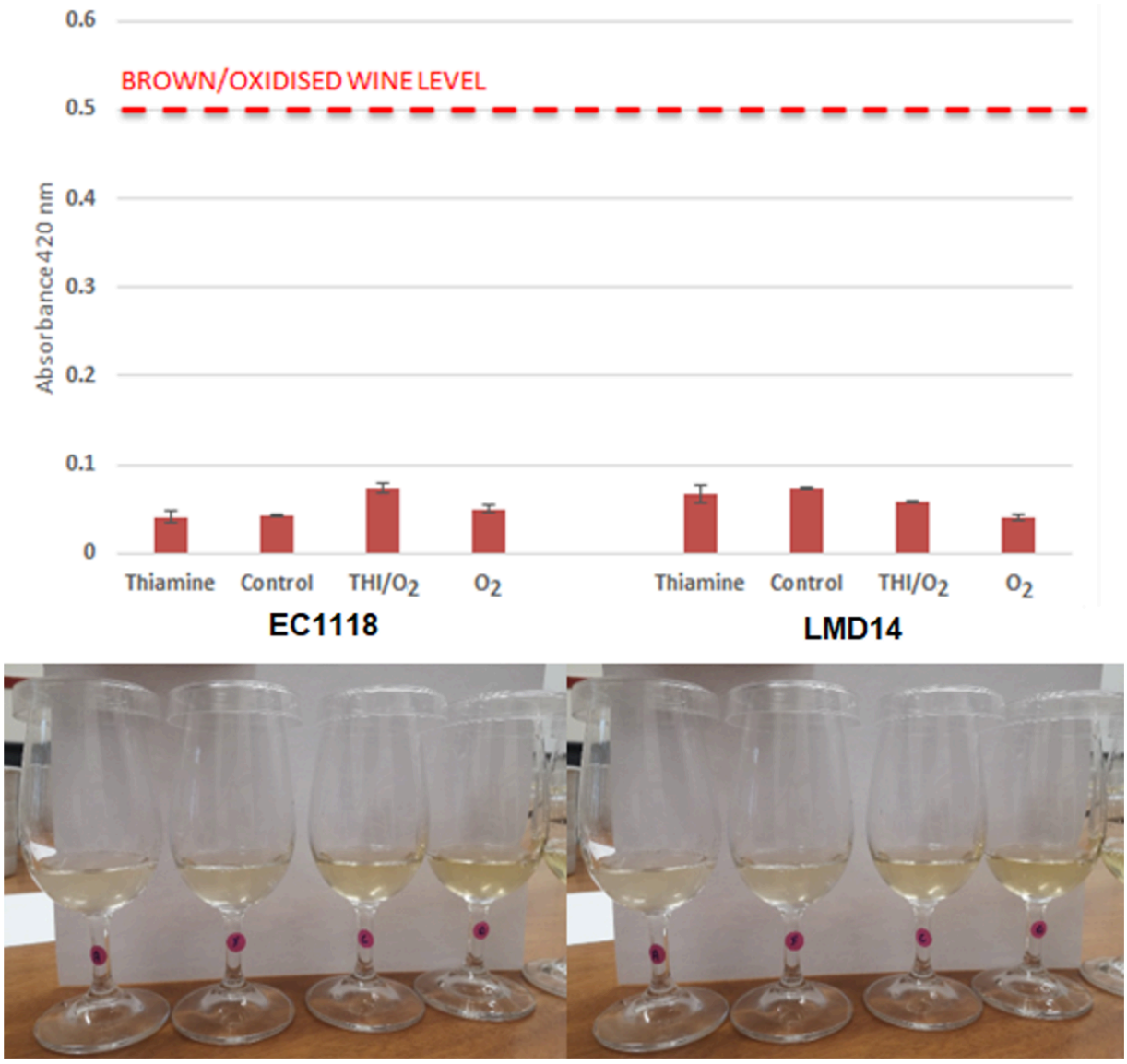


Figure 19: The absorbance of the wine at 420nm was quantified to determine whether oxidation had occurred in the wine.

Finally, to test the impact of nicotinic acid, Chenin Blanc juice was supplemented with 1 mg/L nicotinic acid and 1 mg/L nicotinamide at the start of fermentation (B3+ treatment) as well as added at the time when the strains reached their maximum population (50% treatment). A control fermentation with no addition of vitamin B3 was performed alongside the two treatments. As expected, EC1118 fermented faster than LMD14 and reached the mid-exponential phase fastest. No significant differences were observed in the fermentation kinetics by the addition of vitamin B3 at the start of fermentation nor by the addition at 50% max yeast growth (data not shown). For yeast growth, both treatments for the addition of vitamin B3 resulted in a significantly higher maximum yeast growth for LMD14, whereas EC1118 was unaffected. For the yield in metabolites produced (Table 3), the addition of vitamin B3 did not significantly impact the yield for ethanol or glycerol. However, both supplementation regimes of vitamin B3 did result in a higher yield of acetic acid, however the concentration remained far below the legal limit.

An increase the production of isoamyl alcohol was also observed in both tested strains. No other significant differences were observed in the major volatiles between treatments for each strain.

Table 3: Yields for sugar consumed in each of the tested treatments

| Yields: | EC1118 | | | LMD14 | | |
|---|---|---|---|---|---|---|
| | 1) B3+ | 2) 50%+ | 3) CON | 4) CON | 5) B3+ | 6) 50%+ |
| EtOH (g/g sugar consumed) | 0.47 | 0.48 | 0.48 | 0.52 | 0.53 | 0.52 |
| Acetate (g/g sugar consumed) | 0.0006 | 0.0009 | 0.0008 | 0.0003 | 0.0008 | 0.0007 |
| Glycerol (g/g sugar consumed) | 0.0313 | 0.0318 | 0.0328 | 0.0479 | 0.0617 | 0.0588 |
| Glycerol/Ethanol ratio (%) | 6.59 | 6.60 | 6.83 | 10.35 | 11.74 | 11.26 |

Sensory analysis was performed on the wines generated above. The impact of thiamine supplementation and/or oxygen exposure is summarised in Figure 20.

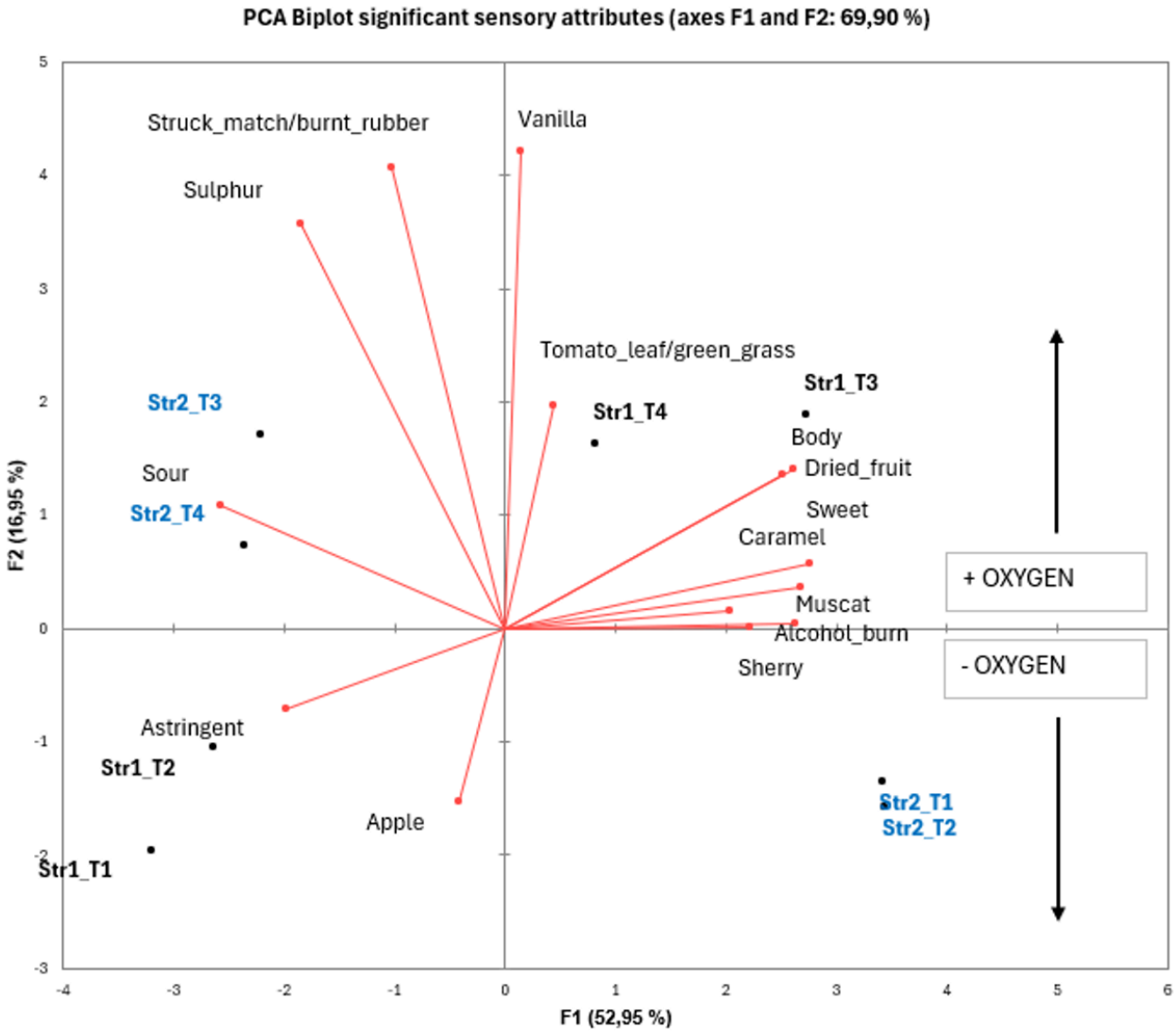


Figure 20: PCA biplot showing the sensory attributes of the Chenin blanc wines obtained from EC1118 (Str 1) and LMD14 (Str 2) in the four treatments (see Table 2).

Clear differences were recorded by the sensory panel between the wines exposed to oxygen and those that were not. However, thiamine supplementation did not significantly impact the wines' sensory profiles. The wines exposed to oxygen did not exhibit any oxidation character. They were not ranked higher or lower but were described as aromatically different.
The supplementation of vitamin B3 did not result in any significant difference (data not shown).

Take-home message: The supplementation of thiamine and vitamin B3 only resulted in minor differences in the wine chemical composition, which did not translate into significant sensory differences. This could be due to the grape juice already containing sufficient concentrations of these vitamins. While deficiency in these vitamins can have a major impact on yeast fermentation performance and metabolic footprint, limited to no impact is observed above a certain threshold. This threshold will need to be established, possibly in a species/strain-dependent manner. However, exposure to oxygen during the yeast growth phase does not oxidise wine (i.e. no browning and no oxidative aroma

characteristics) and has a significant impact on the yeasts' performance and metabolic footprint and ultimately on the wine's sensory profile. Timing and extent of oxygen exposure should be further investigated in different species and strains, but this technique could offer an opportunity to produce wines with altered sensory attributes.

## 8. CONCLUSIONS AND RECOMMENDATIONS

The key findings from the project are summarised in Figure 21.

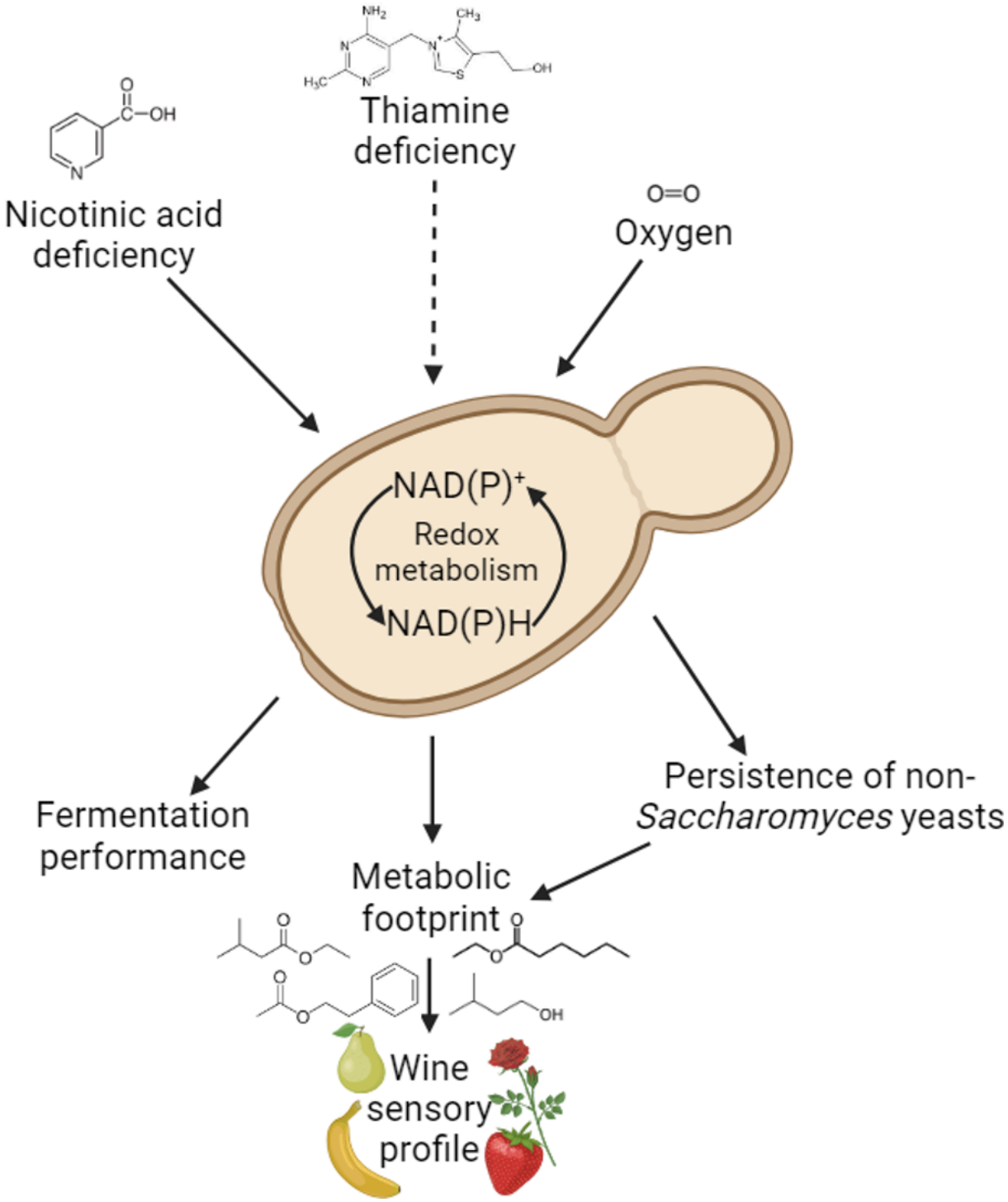


Figure 21: Broad summary of the main outcomes of this project. Direct and indirect impacts are indicated by plain and dashed arrows, respectively.

The results underscore the potential that fine-tuning environmental conditions can have to optimise yeast performance during fermentation. While general rules have emerged regarding how changes in conditions like oxygen levels affect different yeast strains and species, exceptions such as *Starmerella* and *Metschnikowia* species demonstrate that a more nuanced approach is necessary. Future research should focus on precisely adjusting these conditions in the lab, supported by sensory and chemical analysis, to

enhance wine quality in these and other non-*Saccharomyces* yeasts.
Oxygen management emerges as a critical lever in influencing redox balance and fermentation outcomes. However, the exact oxygenation protocols need careful refinement to ensure that weaker fermenters with low $NAD^+$ regeneration capacities can maintain persistence throughout fermentation to impart desirable characteristics. By improving our understanding of these dynamics, particularly in species that struggle with $NAD^+$ regeneration, new strategies can be developed to bolster their fermentative performance.

Finally, while this study primarily examined abiotic factors such as oxygen and nutrient availability, the impact of biotic factors - such as interactions with other yeast species - on redox balance remains unexplored. Future work should investigate how these biotic interactions influence redox dynamics, potentially offering new avenues into mixed-species fermentations.

## 9. PLANNED OUTPUTS

### a) TECHNOLOGY DEVELOPMENT, PRODUCTS AND PATENTS

This project generated new and fundamental knowledge on yeast physiology and biochemistry, with a specific focus on (1) metabolic pathways leading to the production of oenologically relevant compounds, (2) selected environmental conditions governing these pathways and (3) the influence of the genetic background of different yeast species. It helped us understand the impact of redox cofactor management on the production of flavour compounds. It also showed that environmental parameters such as temperature, oxygen and vitamin content influence redox balance and ultimately the production of metabolites. In particular, oxygen appears as an interesting lever to steer metabolism and produce wines with different flavour profiles. Although fine-tuning work is still required, this project was a stepping stone towards making informed decisions on the type and timing of supplementation to apply for a desirable outcome in a species or even strain-dependent manner. The project generated in-depth information on specific commercial and non-commercial yeast strains that can impart valuable characteristics to wine but typically exhibit limited persistence. The information generated in this project will provide relevant information to adjust the formulation of yeast-derived nutrients to maximise the persistence of certain yeast species and ultimately their oenological potential.

### b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

Results from this research project have been disseminated through several high-impact scientific publications, including the International Journal of Food Microbiology, Food Research International and FEMS Yeast Research. These publications have focussed on detailing our findings on redox state management and metabolism in Saccharomyces cerevisiae and other non-Saccharomyces wine yeasts. We have also shared our work with industry partners via semi-scientific articles in IVES Technical Reviews and WineLand, focusing on practical implications for winemakers. Conference presentations, such as the International Congress on Yeasts and the South African

Society for Enology and Viticulture, have facilitated knowledge exchange with the scientific and industry communities. One last popular article is foreseen to summarise some of the most relevant outcomes.

### c) HUMAN RESOURCES DEVELOPMENT / TRAINING (STUDENTS)

| Student Name and Surname | Student Nationality | Degree (eg Hons, MSc) | Level of studies in final year of project | Total Bursary Cost for Industry for entire project |
|---|---|---|---|---|
| **Honours** | | | | |
| Nathalie Dieltjens | South African | BSc | Hons | R 50000 |
| Nathan Naudé | South African | BSc | Hons | R 60000 |
| Julian Pietersen | South African | BSc | Hons | R 60000 |
| Eckart Kassier | South African | BSc | Hons | R 50000 |
| Keegan Clark | South African | BSc | Hons | R 50000 |
| **Masters** | | | | |
| Eckart Kassier | South African | Hons | MSc | R 100000 |
| **PhD** | | | | |
| Viwe Tyibilika | South African | MSc | PhD | R 0 |
| James Duncan | South African | Hons | PhD | R 0 |
| **Postdocs** | | | | |
| Francesca Albonico | Italian | PhD | PhD | R 420000 |

### d) LIKELY PUBLICATIONS (POPULAR, PRESS RELEASES, SCIENTIFIC)

A popular article aimed at winemakers summarising the key outcomes of the project is foreseen.

### e) PRESENTATIONS/PAPERS THAT COULD BE DELIVERED

Scientific Publications:

Tyibilika, V., Setati, M.E., Bloem, A., Divol, B. and Camarasa, C., (2024). Differences in the management of intracellular redox state between wine yeast species dictate their fermentation performances and metabolite production. International Journal of Food Microbiology, 411, 110537. DOI: 10.1016/j.ijfoodmicro.2023.110537

Duncan, J.D., Setati, M.E. and Divol, B. (2024). Nicotinic acid availability impacts redox cofactor metabolism in *Saccharomyces cerevisiae* during alcoholic fermentation. FEMS Yeast Research, 24, foae015. DOI: 10.1093/femsyr/foae015

Duncan, J.D., Setati, M.E. and Divol, B., (2023). Redox cofactor metabolism in *Saccharomyces cerevisiae* and its impact on the production of alcoholic fermentation end-products. Food Research International, 163, 112276. DOI: 10.1016/j.foodres.2022.112276

Duncan, J.D., Setati, M.E. and Divol, B., (Will be accepted after corrections). Oxygen alters redox cofactor dynamics and induces metabolic shifts in *Saccharomyces cerevisiae* during alcoholic fermentation. Food Microbiology

Tyibilika, V., Setati, M.E., Bloem, A., Divol, B. and Camarasa, C., (Will be accepted after corrections) Exploring fermentative metabolic response to varying exogenous supplies of redox cofactor precursors in selected wine yeast species. FEMS Yeast Research

Duncan, J.D., Setati, M.E. and Divol, B., (Submitted August 2024). The cellular symphony of $NAD^+$ metabolism: Redox cofactor management by yeasts during wine fermentation. Applied Microbiology and Biotechnology.

Semi-scientific and Popular Publications:

Duncan, J.D., Ortiz-Julien, A., Setati, M.E. and Divol, B., (2023). Production of flavour compounds by wine yeasts is dependent on the management of their intracellular redox balance. IVES Technical Reviews. DOI: 10.20870/IVES-TR.2023.7725

Duncan, J.D. and Divol, B., (2023). Fruit salad vs varnish – nitrogen sources and their impact on aroma production in wine. WineLand, (August), pp. 57-60.

Duncan, J.D. and Divol, B., (2022). The role of thiamine in sluggish fermentations and crushed dreams. WineLand, (November), pp. 23-24.

Presentations:

Duncan, J.D., Devillers, H., Camarasa, C., Setati, M.E. and Divol, B., (2024). Oral Presentation: “A lesson from *Saccharomyces cerevisiae* in adaptation to aerobic and anaerobic conditions”, International Congress on Yeasts. 29 September-3 October. Cape Town, South Africa.

Tyibilika, V., Setati, M.E., Bloem, A., Divol, B. and Camarasa, C. (2023). Oral Presentation: “Exploring yeast fermentative metabolic response to varying exogenous supplies of redox cofactor substrates”, South African Society for Microbiology. 17-20 September. Stellenbosch, South Africa.

Duncan, J.D., Setati, M.E. and Divol, B., (2023). Oral Presentation: “New insights into vitamin B3 and NAD(H) metabolism in *Saccharomyces cerevisiae* during anaerobic fermentation”, South African Society for Microbiology. 17-20 September. Stellenbosch, South Africa.

Duncan, J.D., Setati, M.E. and Divol, B., (2023). Poster Presentation: “An investigation of redox cofactor management in *Saccharomyces cerevisiae* and the effect on the production of alcoholic fermentation end-products”, South African Society for Microbiology. 17-20 September. Stellenbosch, South Africa.

Tyibilika, V., Setati, M.E., Bloem, A., Divol, B. and Camarasa, C. (2023). Oral Presentation:

“Differences in the management of intracellular redox state between yeast species dictates their fermentation performances and metabolite production.”, South African Society for Enology and Viticulture. 13-14 September. Stellenbosch, South Africa.

Kassier, W.E., Albonico, F., F.F. Bauer and Divol, B., (2023). Oral Presentation: “Strain diversity and timing of lactic acid production in *Lachancea thermotolerans*”, South African Society for Enology and Viticulture. 13-14 September. Stellenbosch, South Africa.

Duncan, J.D., Setati, M.E. and Divol, B., (2023). Oral Presentation: “Vitamin B3 impacts redox cofactors and metabolism in *Saccharomyces cerevisiae* during wine fermentation”, South African Society for Enology and Viticulture. 13-14 September. Stellenbosch, South Africa.

Duncan, J.D., Setati, M.E. and Divol, B., (2023). Poster Presentation: “Investigating the redox cofactor dynamics of *Saccharomyces* spp. In shaping the production of end-products during alcoholic fermentation”, Physiology of Yeasts and Filamentous fungi. 5-8 June. Cork, Ireland.

Thesis defences:

Tyibilika, V., (2024). PhD Dissertation Defence: Exploring redox status management in wine yeast species: Impact on production of flavour compounds. 4 October.

Kassier, W.E., (2023). MSc Thesis Defence: Investigation and characterisation of lactic acid production by Lachancea thermotolerans. 15 November.

Duncan, J.D., (2023). PhD Dissertation Defence: Redox metabolism of *Saccharomyces cerevisiae* alcoholic fermentation: navigating strain, cofactor substrate, and oxygen variability. 5 October.

## 10. PROJECT OUTCOME AND IMPACT

| New Knowledge | Benefits Supply Chain | Direct Grower Application | Direct Packhouse/Winery/ Cellar Application | Other |
|---|---|---|---|---|
| X | | | | |

**Other is:**

**The Value of the project to industry**

This project created new knowledge on the diversity of yeast physiology during fermentation.
It offered an opportunity to optimise fermentation conditions. It also provided insights that can be used to develop improved yeast nutrient formulations, supporting the enhancement of wine quality and fermentation consistency.

## 11. PERSONS PARTICIPATING IN THE PROJECT:

| INITIALS AND SURNAME | HIGHEST QUALIFICATI | RACE | GENDER | INSTITUTE | POSITION | TOTAL COST |
|---|---|---|---|---|---|---|

| | ON | (B,C,I,W) | (M,F,X) | DEPARTM | | TO PROJECT |
|---|---|---|---|---|---|---|
| **RESEARCH PERSONNEL** | | | | | | R 0 |
| B Divol | DSc | W | M | SAGWRI | PL | R 0 |
| ME Setati | PhD | B | F | SAGWRI | co | R 0 |
| C Camarasa | DSc | W | F | INRAE, Montpellier, France | Coll | R 0 |
| A Bloem | PhD | W | F | Montpellier University, France | Coll | R 0 |
| **SUPPORT PERSONNEL** | | | | | | R 173465 |
| Lynn Engelbrecht | MSc | W | F | SAGWRI | TA | R 173465 |

POSITION: Co = Co-worker (other researcher at your institution)
Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

## 12. TOTAL COST OF PROJECT

| TOTAL ANNUAL COSTS (ALL YEARS) | CFPA | RAISINS SA | HORTGRO | SATI | SA WINE | ARC | OTHER | TOTAL |
|---|---|---|---|---|---|---|---|---|
| **2019 and before** | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 |
| **2020** | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 |
| **2021** | R 0 | R 0 | R 0 | R 0 | R 249514 | R 0 | R 285714 | R 535228 |
| **2022** | R 0 | R 0 | R 0 | R 0 | R 464232 | R 0 | R 285714 | R 749946 |
| **2023** | R 0 | R 0 | R 0 | R 0 | R 370579 | R 0 | R 428571 | R 799150 |
| **2024** | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 | R 0 |
| **TOTAL** | R 0 | R 0 | R 0 | R 0 | R 1084325 | R 0 | R 999999 | R 2084324 |